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GRANT NUMBER DAMD17-95-1-5021

TITLE: Molecular Detection of Breast Cancer

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REPORT DATE: February 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
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19980617 053

DTIC QUALITY INSPECTED 1

REPORT DOCUMENTATION PAGE

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Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	February 1998	Annual (9 Jan 97 - 8 Jan 98)

4. TITLE AND SUBTITLE
Molecular Detection of Breast Cancer

5. FUNDING NUMBERS
DAMD17-95-1-5021

6. AUTHOR(S)
Michael F. Clarke, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Michigan
Ann Arbor, Michigan 48109

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, MD 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

Breast cancer is the second leading cause of cancer death among American women, with over 170,000 new cases and 50,000 deaths each year. Despite advances in detection and treatment, mortality from these diseases remains high. Traditional modes of treatment including radiation therapy, chemotherapy, and hormonal therapy have been useful but are limited by the emergence of treatment-resistant cancer cells. Clearly new approaches are needed to treat these diseases. This project is designed to develop novel approaches to detect breast cancer cells that contaminate peripheral blood and bone marrow, and to remove such contaminating cells. An RT-PCR assay has been developed to detect breast cancer cells, and a novel gene therapy vector has been developed to kill contaminating cancer cells. Blood and bone marrow samples obtained from patients with breast cancer are being collected. These samples will be analyzed to determine whether the K19 RT-PCR assay can be used to predict outcome. Next, a gene therapy vector, the bcl-xs adenovirus, has been developed. This vector has promise as a therapeutic agent for the treatment of breast cancer. The work was chosen for a platform presentation at the DOD Breast Cancer meeting in Washington, D.C. in 1997. With additional support from the National Cancer Institute, this virus is undergoing toxicology testing in order to obtain FDA approval for human clinical trials for the treatment of breast cancer.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Metastasis, PCR, Diagnosis, Transplant, Occult, Molecular, Humans, Anatomical Samples, Residual Disease, Bone Marrow, Peripheral Blood, Stem Cells Breast Cancer	102
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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M. T. Clews 2/6/98

PI - Signature Date

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INTRODUCTION

Breast cancer is the second leading cause of cancer death among American women, with over 170,000 new cases and 50,000 deaths each year. Despite advances in detection and treatment, mortality from these diseases remains high. Traditional modes of treatment including radiation therapy, chemotherapy, and hormonal therapy have been useful but are limited by the emergence of treatment-resistant cancer cells. Clearly new approaches are needed to treat these diseases.

One of the more promising approaches for the treatment of metastatic breast cancer is high dose chemotherapy. Breast cancer is susceptible to chemotherapy in a dose-dependent manner. The major dose limiting toxicity of many effective chemotherapeutic agents is hematopoietic toxicity. To overcome this obstacle, autologous bone marrow or peripheral blood stem cells are harvested from the patient prior to the administration of the high dose chemotherapy, and then reinfused after the chemotherapy has been excreted and/or metabolized. Although the initial clinical trials of such strategies are promising, there are other obstacles that need to be overcome to optimize results. The agents used for the systemic treatment of this disease need to be improved. Additionally, bone marrow transplantation for solid tumors such as breast cancer is complicated by the fact that these cancers frequently metastasize to the bone marrow.

To date, two significant advances have resulted from this proposal. A sensitive assay has been developed to identify breast cancer cells in the bone marrow and the peripheral blood. Next, an adenovirus vector has been developed that is selectively lethal to breast cancer cells, but not normal cells. The results of preclinical studies with this virus have been successful. The National Cancer Institute (NCI) has decided to hold an IND for this virus, and toxicology testing is underway in order to obtain FDA approval for clinical trials. This virus should enter clinical trials for the treatment of breast cancer sometime in the next two years.

BACKGROUND

The use of autologous bone marrow transplant (BMT) as a part of cancer treatment has increased dramatically in recent years. For example, autologous BMT often is used to protect patients from the hematopoietic toxicity of high dose chemotherapy in the treatment of breast tumors. However, the success rate for this treatment regimen is jeopardized by contamination of the autologous marrow with low numbers of tumor cells. Thus, the ability to selectively kill or purge contaminating tumor cells in the marrow *in vitro,* prior to autologous transplant, could significantly improve the chances of long term survival.

The most common technique of purging tumor cells from bone marrow relies upon monoclonal "anti-tumor" antibodies to immunologically remove tumor cells from a suspension of marrow cells. However, the success of this approach depends on the availability of well characterized antibodies capable of detecting each tumor cell phenotype. This method of purging rarely removes all tumor cells, and requires repeated purging cycles which can decrease hematopoietic cell viability. Moreover, the specific antigens are rarely "tumor cell specific", often being found on normal cells, albeit at low antigen density. Other purging methods, such as photo-sensitization agents or chemotherapy, also can cause significant hematopoietic toxicity.

Until recently, investigators thought that cancer treatments based on chemotherapy, or radiation therapy, exerted their tumor-killing specificities based on the different sensitivities of

rapidly dividing (versus quiescent) cells to these agents. Recent evidence suggests that radiation and many chemotherapy agents specifically kill tumor cells, while sparing normal cells, by triggering of the programmed cell death (PCD) pathway. The induction of PCD is impeded by genes such as *bcl-2*, and its presence in cancer cells thus reduces the efficiency of conventional cancer therapy. Mechanistically, *bcl-2* does not appear to be mitogenic or transforming, but it cooperates with c-myc, and members of the ras family, to cause transformation. Additionally, *bcl-2* acts to inhibit apoptosis induced by p53, *myc*, chemotherapy, and ionizing radiation. Members of this gene family have been implicated in the progression of a large number of human solid tumors, including lymphomas, cancers of the breast, lung, and prostate, as well as neuroblastoma. These observations suggest that suppression of *bcl-2* expression using gene therapy methods would be a valuable tool in cancer treatment, by increasing the susceptibility of tumor cells to existing chemotherapeutic and radiation treatments.

Other members of the *bcl-2* gene family have recently been isolated and partially characterized. A *bcl-2* homologue, *bcl-x*, gives rise to two mRNA species through alternative splicing. One of these, *bcl-x_L*, functions in a manner similar to *bcl-2*, and inhibits apoptosis. The other, *bcl-x_S*, functions as a repressor to *bcl-2* and acts to promote apoptosis. We recently demonstrated that adenoviral-mediated overexpression of *bcl-x_S* inhibited the anti-apoptotic role of *bcl-2* and induced PCD in a variety of primary tumors and tumor cell lines. This PCD was augmented by, but not dependent upon, the tumor suppresser p53. In contrast, normal bone marrow hematopoietic stem cells resisted *bcl-x_S*-adenovirus induced PCD apoptosis. We postulated that the resistance of hematopoietic stem cells to the *bcl-x_S* adenovirus was due to the lack of expression of adenovirus transgenes in hematopoietic stem cells or alternatively to the inability of *bcl-x_S* to induce cell death in these cells. We report that murine hematopoietic stem cells resist expression of an adenovirally transduced gene. Moreover, a *bcl-x_S* adenovirus works synergistically with a Herpes virus TK adenovirus to specifically purge tumor cells from *in vitro* hematopoietic cultures, with the preservation of transplantable stem cells. Thus, these results indicate that adenovirus vectors fail to transduce genes into early hematopoietic stem cells. Therefore, this suggests that adenovirus vectors encoding suicide genes such as *bcl-x_S* or Herpes virus TK would preferentially kill the contaminating tumor cells derived from epithelial tissues found in bone marrow cell populations, thus serving as an excellent means of marrow purging.

Significant progress in the purging of breast cancer cells has been made. We have further defined the use of adenovirus suicide vectors for killing breast cancer cells that contaminate the bone marrow of patients with breast cancer. Last year, we found that a pure population of mouse hematopoietic stem cells are not transduced by an adenovirus vector. We have extended this observation and now show that human hematopoietic stem cells are also not transduced by such vectors. The National Cancer Institute has now decided to do toxicology testing of the *bcl-xs* adenovirus to obtain FDA approval for use of the virus in human clinical trials. We envision such trials to begin within the next two years.

We have made marked progress since last year. Specifically, we have developed preclinical animal models to test the potential utility of the *bcl-xs* adenovirus for use in the treatment of breast cancer. Not only has the virus been used in bone marrow purging preclinical trials, but also for systemic treatment. This work was chosen for a platform presentation at the 1997 DOD Breast Cancer meeting in Washington, D.C. Abstracts of the relevant manuscripts follow. These abstracts are from manuscripts that have been published or submitted relevant to this grant. Note that the manuscripts are included as an appendix to this report. The manuscripts serve as the methods and figure sections of this report.

BODY

Significant progress in completion of the goals of this grant have been made in the first two years.

In general, progress has been made on all of the original tasks. However, due to developments since the original grant was submitted, we have concentrated on certain tasks and made progress beyond the original goals. Other tasks have been modified due to recent developments in the field. Progress on each task is given. The clarification requested by the reviewer of last year's progress report are given. When tasks have been modified, the reasons for modification are given.

One point needs to be emphasized. The reviewer states that most progress has been made in regards to the purging strategy, and less in the K19 analysis of patients with breast cancer. This, in fact, is not true. The initial analysis of the usefulness of K19 RT-PCR to detect breast cancer in the blood and bone marrow was published by Dr. Mark Roth, the original P.I. of this project. Since then, there have been over 120 publications worldwide that expand upon the original data. In addition, there is now a national clinical trial (SWOG 9702) that examines the prognostic significance of K19 detection in women undergoing high dose chemotherapy. Due to these new events, coupled with the fact that no single institution can collect as many samples as a national clinical trial can, we have elected to enter our patients in the national trial. Thus, some of the original tasks have been modified to reflect the marked success of this assay.

Although others are now also using this assay, we continue to collect samples here at the University of Michigan and analyze them for K19 expression.

Task one, to test the hypothesis that women with poor prognostic indicators are more likely to present with the presence of blood and/or bone marrow micrometastases, is ongoing with sample collections.

-1A. Sample collection and PCR assays. To date, we have collected blood samples from 123 patients including 20 patients with stage I or II cancer, 22 with stage III, and 123 with stage IV breast cancer. We plan to increase the number of samples obtained from patients with the stage I, II, and III breast cancer. We have begun to analyze specimens for K19 expression. RT-PCR assays of the first 13 specimens are being done at this time.

-1B. Clinical follow-up. Follow-up of the outcome of stage I, II and III prognosis patients will begin when more samples are obtained from this group of patients. See enclosed informed consent and IRB approval for continued sample collection. We plan on collecting more samples, and will begin to analyze outcomes after collecting samples from ~10 more patients.

Task two, to use a PCR based assay to detect the mammary cell specific keratin-19 mRNA and evaluate the presence of occult breast cancer cells in patients undergoing BMT, is progressing well, indeed ahead of schedule, and two manuscripts have been published that address this task. As stated previously, there is now a SWOG national clinical trial to determine the prognostic significance of K19⊕ cells in women undergoing BMT. The University of Michigan is a SWOG member, and we will enter our patients into the national study rather than a limited local trial.

-1A. To determine the relative frequency of tumor contamination of marrow versus peripheral blood stem cell harvests. To date, we have collected samples from 59 patients that have undergone BMT. Forty seven samples are from peripheral blood stem cell harvests, and 13 are from bone marrow harvests (one patient had both aphoresis and bone marrow). To date, 20% of the peripheral blood samples have been positive, and 46% of the bone marrow samples have been positive. At the time this grant was written, bone marrow harvest was the source of the hematopoietic cells used for rescue from high dose chemotherapy. Now, peripheral blood stem cell harvests are used exclusively for rescue from high dose chemotherapy. This task has not been published. This task was accomplished using standard published techniques, and thus submission of a manuscript is not planned.

-1B. Correlation of PCR results with clinical outcome. There are not yet enough samples obtained from patients with stage I-III breast cancer. This is also the case with breast cancer patients undergoing high dose chemotherapy. The positive rate in peripheral blood stem cell harvests is lower than that seen in bone marrow. Since peripheral blood stem cell harvests are used, only about 20% of the patients are K19 positive. Only about 20% of such samples are PCR positive for cancer. Therefore, the sample size is still too small to make any clinical correlation's at this time. As stated before, we will now enter our patients into the SWOG 9702 national trial.

-1C. Evaluate the efficacy of bone marrow and stem cell culture purging techniques to eliminate breast cancer cells.

We have published a manuscript describing a novel method for purging contaminating cancer cells from bone marrow hematopoietic stem cells. Many cancers overexpress a member of the *bcl-2* family of inhibitors of apoptosis. To determine the role of these proteins in maintaining cancer cell viability, an adenovirus vector that expresses *bcl-x$_S$*, a functional inhibitor of these proteins, was constructed. Even in the absence of an exogenous apoptotic signal such as x-irradiation, this virus specifically and efficiently kills carcinoma cells arising from multiple organs including breast, colon, stomach, and neuroblasts. In contrast, normal hematopoietic progenitor cells, and primitive cells capable of repopulating immune-deficient SCID mice, were refractory to killing by the *bcl-x$_S$* adenovirus. This vector may prove useful in killing cancer cells contaminating the bone marrow of patients undergoing autologous bone marrow transplantation. (appendix).

One of the issues raised in the review of last year's review was that we have not done any preclinical animal models. This issue has been addressed. Several animal model studies have now been completed using *bcl-xs* adenovirus. Abstracts of these studies follow this section. The manuscripts resulting from these studies are included. Furthermore, we have purified *bcl-xs* adenovirus in the University of Michigan Human Applications Lab (HAL) facility. This virus is clinical grade material that can be used in clinical trials. We are in the process of producing enough clinical grade material in order to send it to the NIH. This virus will be used for toxicology testing for the FDA.

As stated previously, there is now a SWOG national clinical trial to determine the prognostic significance of K19⊕ cells in women undergoing BMT. The University of Michigan is a SWOG member, and we will enter our patients into the national study rather than a limited local trial.

Task 3. Develop additional markers for molecular detection of occult breast carcinoma.

-3A. Evaluate the specificity and sensitivity of PCR based detection of other mammary specific RNA sequences. No new markers have been detected. The K19 marker has been both sensitive and specific. Any new PCR detection strategies will be based on the identification of novel genes found in tasks 3C and 3D.

-3B. Develop non-radioactive detection schema. A nested primer approach was used to detect K19 cDNA (see appendix, manuscript #1). PCR experiments using K19 primers labeled with either 6FAM or HEX fluorescent markers so that the PCR product can be detected using an automated sequencer. The PCR products were analyzed with an automatic sequencer. Various amounts of MCF-7 breast cancer cell RNA was mixed with normal bone marrow RNA (from 1x 10^6 cells). These mixing experiments showed that after 2 rounds of PCR we were able to detect K19 mRNA in two of three samples containing 10 MCF-7 cells, and two of four samples containing 1 MCF-7 cell. K19 was not detected in normal peripheral blood. This task is completed; a non-radioactive detection schema has been developed. Either nested PCR with gel electrophoresis, or with fluorescent detection using an automated sequencer, can be used to detect the K19 mRNA. This task has not been published. This task was accomplished using standard published techniques, and thus submission of a manuscript is not planned.

-3C. Detection of novel breast cancer peptides. Breast cancer cDNA has been made from RNA isolated from SUM 159 human breast cancer cells. This cDNA will be used to generate a library to identify novel genes.

-3D. As described in the proposal, screening for novel breast cancer peptides will begin this year.

In order to identify cell surface proteins expressed in breast cancer cells, we have developed a novel cDNA cloning vector. Many cell surface proteins have a "leader sequence" at the amino terminus. This sequence directs the transport of a protein to the outer cell membrane, and then is cleaved. We constructed a cloning vector that contains a CD33 gene with the leader sequence deleted. The 5' portion of the cDNA library was fused to the CD33 vector. The vector was transfected into COS7 cells, which do not express CD33. Thus, only cells that are transfected with a CD33 vector that has a cDNA encoding a protein with a leader sequence express a fusion protein that inserts CD33 into the COS7 cell membrane. The CD33 and COS7 cells are isolated and then the plasmid is rescued (Figure 1). To date, we have sequenced twenty-three plasmids identified in this manner. Although several genes encoding known cell surface proteins have been identified, no novel proteins have yet been identified.

A bcl-xs adenovirus selectively induces apoptosis in transformed but nor normal mammary cells.

Abstract

Oncogenes which drive the cell cycle such as c-myc, can sensitize cells to apoptosis upon growth factor withdrawal. This suggests the possibility that during carcinogenesis, the overexpression of genes such as bcl-2 or bcl-x_L are required to inhibit apoptois induced by oncogene expression. We hypothesized that inhibition of Bcl-2/Bcl-xL by the proapoptotic Bcl-xS protein would result in selective induction of cell death in carcinoma cells compared to non-transformed cells. In order to test this hypothesis and to determine the therapeutic efficacy of a bcl-x_s adenovirus, we compared the effects of Bcl-x_S expression delivered by the bcl-x_s adenoviral vector, on non-transformed murine mammary cells (Comma-1D) and a c-myc transformed mouse

mammary cell line (Myc-83). We found that whereas the non-transformed Comma-1D cells are resistant to the effects of the bcl-x_s vector, this vector efficiently induced apoptosis in the Myc-83 cells; consistent with the hypothesis that inhibition of Bcl-2 family of genes can result in selective killing of cancer cells compared to their non-transformed counterparts.

We extended these studies to a mouse breast cancer ascites model by demonstrating that the bcl-x_s adenoviral vector introduced intraperitoneally, reduces ascite formation and significantly prolongs survival with no detectable toxicity to normal mouse tissues. These studies demonstrate that the bcl-x_s adenovirus selectively kills transformed cells in vitro and in vivo, supporting the use of this approach for the gene therapy of breast cancer.

A recombinant *bcl-x_s* adenovirus selectively induces apoptosis in cancer cells but not in normal bone marrow cells.

Abstract

Many cancers overexpress a member of the *bcl-2* family of inhibitors of apoptosis. To determine the role of these proteins in maintaining cancer cell viability, an adenovirus vector that expresses *bcl-x_s*, a functional inhibitor of these proteins, was constructed. Even in the absence of an exogenous apoptotic signal such as x-irradiation, this virus specifically and efficiently kills carcinoma cells arising from multiple organs including breast, colon, stomach, and neuroblasts. In contrast, normal hematopoietic progenitor cells and primitive cells capable of repopulating severe combined immunodeficient mice were refractory to killing by the *bcl-x_s* adenovirus. These results suggest that Bcl-2 family members are required for survival of cancer cells derived from solid tissues. The *bcl-x_s* adenovirus vector may prove useful in killing cancer cells contaminating the bone marrow of patients undergoing autologous bone marrow transplantation.

Bcl-2 protects murine erythroleukemia cells from p53-dependent and -independent radiation-induced cell death.

Abstract

To better understand the molecular basis of radiation-induced cell death, we studied the role of the *bcl-2* oncogene and the *p53* tumor suppressor gene in this process. A temperature-sensitive mutant of murine p53 ($p53^{Val\text{-}135}$) and/or *bcl-2* was transfected into murine erythroleukemia cells (MEL, DP16-1, which are null in p53). We demonstrate that radiation-induced cell death occurs by both p53-dependent and -independent pathways and overexpression of *bcl-2* modulates both pathways. When viability was measured 24 h post-radiation, cells that had been briefly exposed to wtp53 immediately after X-ray irradiation had decreased survival as compared to the unirradiated cells expressing wtp53 or X-ray irradiated DP16-1 cells. However, at later times X-ray irradiated parental DP16-1 cells also had decreased survival compared to the unirradiated control. This decrease in survival began 48 h following radiation. Bcl-2 prevented radiation-induced cell death in DP16-1 cells expressing wtp53 and delayed radiation-induced cell death in DP16-1 cells without wtp53. X-ray irradiated cells expressing wtp53 displayed microscopic and biochemical characteristics consistent with cell death due to apoptosis. DP16-1 cells which were untransfected or co-transfected with wtp53 and *bcl-2* displayed characteristics of cells undergoing necrosis. These results suggest that radiation-induced cell death occurs by both p53-dependent and p53-indepedent pathways. The p53-dependent pathway results in cell death via apoptosis and occurs approximately 24 h following radiation. The p53-indepenent pathway does not appear to involve

apoptosis and occurs at a later time, starting 48 h after X-ray exposure. Thus, bcl-2 protects cells from p53-dependent radiation-induced apoptotic cell death and attenuates p53-independent radiation-induced cell death.

bcl-x_s gene therapy induces apoptosis of human mammary tumors in nude mice

Abstract

Bcl-x_s is a dominant negative repressor of Bcl-2 and Bcl-x_L, both of which inhibit apoptosis. We used a replication-deficient adenoviral vector to transiently overexpress Bcl-x_s in MCF-7 human breast cancer cells, which overexpress Bcl-x_L. Infection with this vector induced apoptosis *in vitro*. We then determined the effects of intratumoral injection of *bcl-x_s* adenovirus on solid MCF-7 tumors in nude mice. Tumors injected four times with the *bcl-xs* adenovirus showed a 50% reduction in size. Using terminal transferase-mediated dUTP-digoxigenin nick end labeling, we observed apoptotic cells at sites of *bcl-x_s* adenoviral injection. These experiments demonstrate the feasibility of using *bcl-x_s* gene therapy to induce apoptosis in human breast tumors.

Sensitive detection of occult breast cancer by the reverse-transcriptase polymerase chain reaction

Abstract

Detection of occult carcinoma in patients with breast cancer may aid the establishment of prognosis and development of new therapeutic approaches. To improve on existing methods of detection, we have developed a reverse-transcriptase polymerase chain reaction (RT-PCR) assay for keratin 19 (K19) transcripts to identify mammary carcinoma cells in the peripheral blood and bone marrow of patients with breast cancer. Peripheral-blood or bone marrow samples obtained from 34 patients with stages I to IV breast cancer and 39 control subjects without breast cancer were screened for K19 mRNA by nested primer PCR. In reconstitution experiments, K19 RT-PCR reliably detected 10 mammary carcinoma cells in 1 million normal peripheral-blood mononuclear (PBMN) cells. Four of 19 patients with stage IV breast cancer had detectable K19 transcript in peripheral blood. Five of six patients with histologically negative bone marrow biopsies following preablative chemotherapy and before autologous bone marrow transplant (BMT) were positive by this assay. Stem-cell apheresis harvests obtained from one of these patients and three additional patients immediately before BMT were all K19-negative. K19 RT-PCR analysis of CSF from a breast cancer patient with known carcinomatous meningitis was also positive. Thirty-eight of 39 non-breast cancer patients had negative K19 RT-PCR assays. The one exception was a patient with chronic myelogenous leukemia. RT-PCR of K19 is a sensitive, specific, and rapid method for detection of occult mammary carcinoma cells in the peripheral blood and bone marrow of patients with breast cancer. The presence of residual breast cancer cells in histologically normal bone marrow aspirates but not in stem-cell apheresis harvests is a frequent finding. This assay may be useful in diagnosing metastatic disease, as well as in monitoring the effectiveness of systemic therapy.

METHODS

The manuscripts included in the appendix serve as the methods section and contain a relevant bibiography.

CONCLUSIONS

Significant progress has been made in completing the tasks of this proposal. Keratin 19 appears to be a novel and effective marker for RT-PCR detection of breast cancer cells in peripheral blood and the bone marrow. The collection of patient samples now exceeds one hundred RNA preparations. This includes patients with stage I through IV breast cancer, and includes more than fifty patients that have undergone BMT.

As an adjunct to surgery, radiation, or chemotherapy, autologous bone marrow transplants (BMTs) are increasingly used as a method to increase survival of patients with aggressive non-hematopoietic tumors. However, retroviral tagging and PCR studies indicate that autologous marrow is often the source of cancer relapse in these patients. Several methods have been devised to purge marrow of tumor cells prior to transplantation, but each has distinctive shortcomings. Immunologic methods depend on a unique tumor cell surface epitope and a high avidity antibody for efficient negative selection. Chemical techniques can have significant hematopoietic toxicity. We have previously shown that adenovirally mediated transient expression of *bcl-x$_S$*, a functional repressor of *bcl-2*, would induce PCD in contaminating tumor cells found in bone marrow cell preparations. It is reported here that a pure population of hematopoietic stem cells does not express a transgene when exposed to a recombinant adenovirus. We further postulated that hematopoietic stem cells would retain the ability to repopulate hematopoiesis following treatment with adenoviral vectors.

We show that a combination of *bcl-x$_S$* and TK adenovirus are the most effective and least toxic method of killing MCF-7 cells in a mixed tumor/hematopoietic *ex vivo* culture. Of all tumor cell types we have tested to date, MCF-7 cells have proven to be among the most resistant to *bcl-x$_S$* adenovirus treatment. By combining a TK adenovirus/gancyclovir treatment with *bcl-x$_S$* adenovirus infection of mixed tumor/marrow cultures, MCF-7 cells are purged from the marrow to below detectable levels. In addition, the combination TK/*bcl-x$_S$* treatment results in an equally efficient purging of hematopoietic progenitors compared to an equivalent treatment using only *bcl-x$_S$*, while resulting in a progenitor cell toxicity that is equal or slightly less. This combined treatment minimizes the non-specific hematopoietic toxicity of these adenoviral vectors, while preserving MCF-7 purging efficiency, although TK treatment alone may be a useful treatment. This data thus supports the hypothesis that *bcl-x$_S$* overexpression mediated by adenoviral vectors may be used to effectively purge solid tumor cells from human bone marrow. Although the mechanism behind this observation is unclear, to date MCF-7 cells have proven to be the only cell type for which a combined *bcl-x$_S$*/TK infection is required to completely purge human marrow *in vivo*. Other cancer cell lines, such as the SHEP 1 neuroblastoma line, are efficiently purged by *bcl-x$_S$* viral MOIs that do not have any significant effect on hematopoietic cell activity.

In this work, hematopoietic stem and progenitor cells are shown to exhibit resistance to greater MOIs than that required to infect 100% of neuroblastoma cells with an adenovirus marker gene. Previous work has shown that mouse hematopoietic stem cells can be isolated on the basis of the phenotype Thy-1.1^{lo} Lin$^-$ Sca-1^{+30}. As few as 30 of these cells can rescue 100% of lethally irradiated mice, producing long-term, multilineage reconstitution. By purifying homogeneous populations of murine stem cells, we were able to directly target them *in vitro* with adenovirus at MOIs greater than those necessary to kill tumor cells. After such treatment, murine stem cells retained their viability but did not express the adenoviral LacZ gene, whereas tumor cells expressed this marker. Confirming and extending this data, our *in vivo* studies show that murine marrow

infected with bcl-x_S, TK, or LacZ adenovirus retains the capacity for long term, apparently multilineage, engraftment upon transplant into lethally irradiated syngeneic mice.

The ability to selectively kill tumor cells, while sparing all of the hematopoietic cells in bone marrow prior to autologous transplantation, represents a novel method in purging/transplantation as a treatment of many human neoplasms. Previous methods involving immunologic, mechanical, or chemical based tumor purging have had limited success, required extensive marrow processing, or were useful for treatment of only one specific cell type. We conclude that bone marrow purging using an adenoviral-based method (that can be used alone or in conjunction with other purging strategies) represents a simple, quick, and efficient method for purging a wide variety of non-hematopoietic tumor cells while retaining hematopoietic stem cell activity.

New data obtained from animal models has demonstrated that the *bcl-xs* adenovirus may prove useful for *in vivo* treatment of breast cancer. We have purified clinical grade virus in the Human Applications Lab. After production of sufficient virus, it will be sent out to the NIH for toxicology testing. We hope to obtain IND from the FDA within 1-2 years.

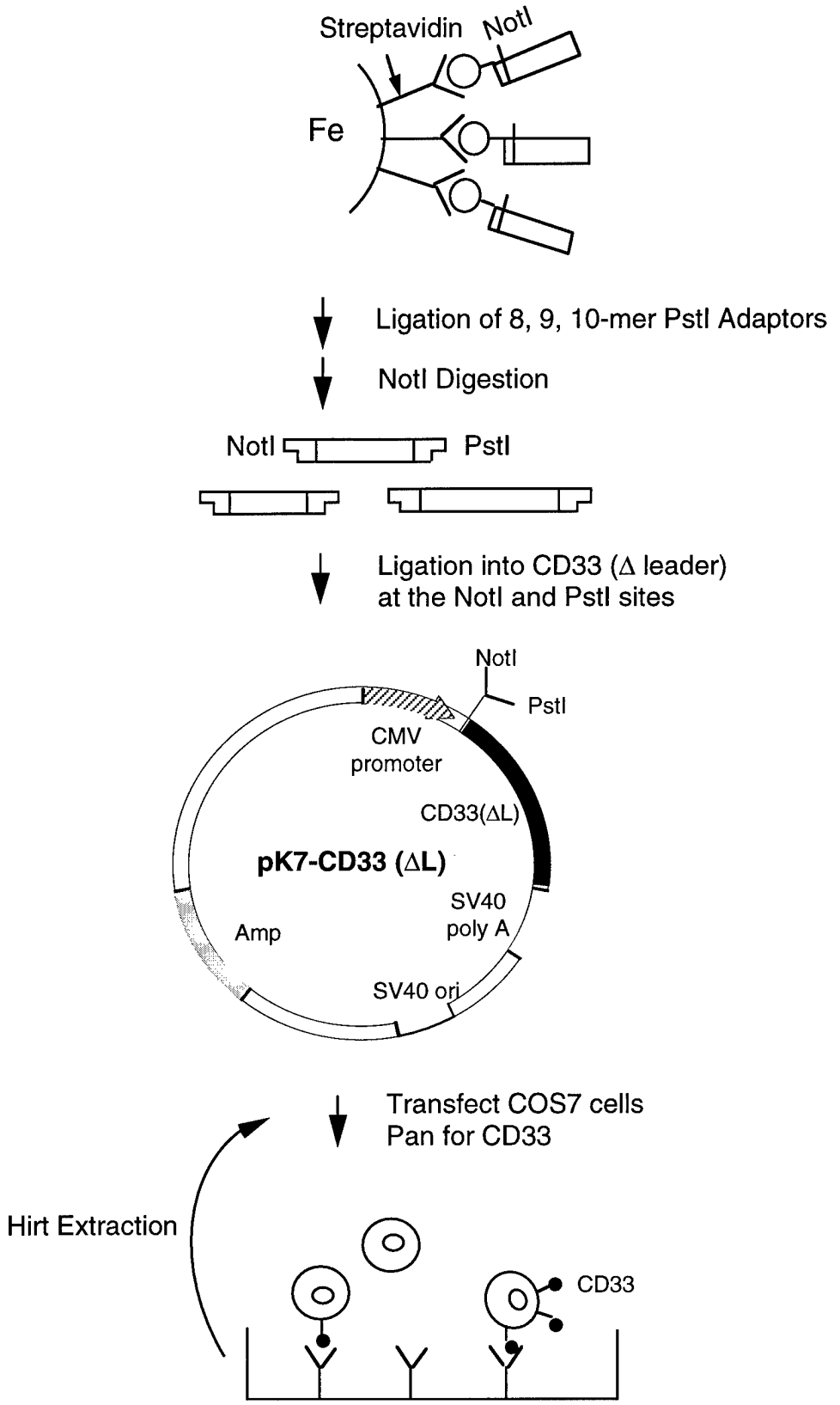
The *bcl-xs* adenovirus will begin toxicology testing for the FDA this year. We anticipate clinical breast cancer clinical trials to begin with this virus within one to two years.

Finally, a human breast cancer cDNA has been made and will be used to attempt to identify novel peptides expressed by breast cancer cells. Any such peptides identified will be used to develop new diagnostic markers for breast cancer.

FIGURE LEGEND

Figure 1. The cell surface protein gene trap strategy is shown.

Cell Surface Protein Trap



A method of limited replication for the efficient *in vivo* delivery of adenovirus to cancer cells

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Running Title: Limited replication of adenovirus.

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ABSTRACT

Replication deficient viral vectors are currently being used in gene transfer strategies to treat cancer cells. Unfortunately, viruses are limited in their ability to diffuse through tissue. This makes it virtually impossible to infect the majority of tumor cells *in vivo* and results in inadequate gene transfer. This problem can be addressed by allowing limited viral replication. Limited viral replication facilitates greater penetration of virions into tissue and can improve gene transfer. We have developed a strategy of limited viral replication using AdRSVlaclys, a chemically modified E1-deleted adenovirus, to co-deliver an exogenous plasmid encoding the adenovirus E1 region. This system allows one round of viral replication. We examined the effect of this limited adenovirus replication *in vitro* and *in vivo*. In culture, co-delivery of virus and pE1 resulted in a large increase of infected cells when compared to control cells exposed to virus and pUC19. In experiments on nude mice bearing Hela ascites tumors, intraperitoneal injection of AdRSVlaclys/pE1 resulted in a significantly higher percentage of infected Hela cells as compared to the PBS controls ($p < 0.05$) or the AdRSVlaclys/pUC19 controls ($p < 0.01$). These data demonstrate that the transcomplementation of replication deficient adenovirus with exogenous E1 DNA leads to limited replication, and this controlled replication enhances gene transfer efficiency of adenovirus *in vivo*.

OVERVIEW SUMMARY

Replication defective viral vectors are limited in their ability to diffuse through tissue. This poses a problem for treating tumors *in vivo* using gene transfer. The following paper demonstrates that limited replication of adenovirus leads to greater gene transfer efficiency *in vitro* and *in vivo* without introducing additional safety concerns beyond traditional adenovirus administration. This has implications for the improvement of current gene transfer methods for treating cancer.

INTRODUCTION

Viral vectors are among the most efficient vehicles for gene transfer *in vitro* and *in vivo* (Mulligan, 1993). For this reason, replication deficient viral vectors have been used in various gene transfer approaches to treat cancer cells (Crystal, 1995). Unfortunately, this approach is limited in that it is nearly impossible to infect the majority of tumor cells *in vivo* due to physical constraints imposed by both the virus and tumor. Tumors *in vivo* are usually present as solid masses or sheets many layers thick, as opposed to the easily infected monolayers in cell culture. Typical virions are large enough to prevent significant diffusion through these cell layers. In addition, in some *in vivo* animal models the amount of virus administered is limited by the volume that can actually be physically injected.

The limitations in vector delivery presents a serious problem in targeting cancerous cells *in vivo* using gene transfer. Delivery of a cytotoxic or tumor suppressing virus may temporarily slow down tumor growth, but is doomed to ultimately fail if some tumor cells are left unharmed. Even strategies with a "bystander effect" (such as the herpes simplex virus *thymidine kinase* gene) require that a significant amount of tumor cells are infected, and most successful animal models of *thymidine kinase* delivery have involved the administration of retroviral producer cell lines to ensure that this is the case (Culver et al., 1992; Takamiya et al., 1992; Barba et al., 1994).

It may be possible to circumvent viral delivery problems with controlled replication of a viral vector *in vivo* (Goldsmith, 1994). This could allow greater penetration of the virus beyond the first several cell layers (see Fig. 1). Limited replication would also increase the effective local viral titer. In order for controlled

replication to occur, the genes necessary for viral replication must be co-delivered with the viral vector. However, these genes must not be engineered into the viral genome or the result would be a fully replication competent virus. We have developed a model of controlled *in vivo* replication using adenovirus. Adenovirus is relatively chemically stable and can be manipulated by adding polylysine without completely destroying infectious activity (Wagner et al., 1992; Cristiano et al., 1993; Fisher and Wilson, 1994). This allows exogenous DNA to bind to the modified adenovirus by electrostatic interactions and piggyback its way into infected cells. By using this method to co-deliver a plasmid encoding the adenovirus E1 region, standard E1-deleted adenovirus can enter a round of replication. We will refer to this method as limited replication, because barring recombination events, all of the progeny of this process will be replication deficient, eliminating virus replication after one round. Adenovirus also has other favorable properties for use in this system. Adenovirus can be produced at high titers and infect a broad range of dividing and non-dividing cells (Kozarsky and Wilson, 1993). In addition, adenovirus has a lytic life cycle, which lyses the cell after virus replication is complete and thus may expose more tumor surface area for further gene transfer.

Other viral vectors all have one or more deficiencies for use in such a system. Retrovirus integrates into the host genome and will continually produce virus for the life of the cell (assuming the transgene is not lethal). Both of these properties are safety concerns (Varmus, 1988). Retrovirus is also surrounded by a relatively unstable lipid bilayer that is difficult to chemically modify without completely abolishing biologic activity (Rosenberg et al., 1997). Herpes simplex

virus has a similar bilayer and presents possible toxicity concerns (Glorioso et al., 1997). Adeno-associated virus (AAV) cannot replicate without adenovirus (Fisher et al., 1997), which defeats the purpose of using AAV in the first place. Therefore, adenovirus appears to be the ideal choice for this system.

Here we demonstrate that transcomplementation of replication deficient adenovirus with exogenous E1 DNA leads to limited viral replication. We have constructed a simple system in which adenovirus/plasmid DNA complexes can be used to achieve this effect *in vitro*. Finally, we have extended this study to demonstrate enhanced gene delivery in a relevant *in vivo* model. This addresses a major problem in current approaches towards cancer gene therapy.

MATERIALS AND METHODS

In vitro limited replication of adenovirus using liposomes

Construction of AdRSVlacZ has been described (Davidson et al., 1994). The plasmid pE1, which contains the E1A and E1B regions of adenovirus (nucleotides 1-5778), was a gift from Eileen White (White and Cipriani, 1989). This plasmid, as well as all plasmids described hereafter, was purified by centrifugation through a CsCl/EtBr gradient (Sambrook et al., 1989). Hela cells (human cervical carcinoma) were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% Fetal Bovine Serum (FBS) and 1% P/S (100 international units/ml of penicillin and 100 µg/ml of streptomycin). SKOV3 (human ovarian carcinoma) cells were maintained in McCoy's 5A Medium containing 10% FBS and 1% P/S.

Cells were seeded in 6-well dishes and allowed to reach 80% confluence. Lipofectamine (Gibco BRL) was used to lipofect either 1 µg of pUC19 or 1 µg of pE1 into each well. Lipofection procedures were done as recommended by the manufacturer. After 24 hours at 37°C, the medium was replaced with 2 ml of fresh medium (2% FBS, 1% P/S) containing 1 x 10^6 AdRSVlacZ viral particles. Each well was fed with 2 ml of medium (2% FBS, 1% P/S) at day three and stained on days six or eight with 5-bromo-4-chloro-3-indolyl β-D-galactosidase (X-gal). Before staining, cells were fixed in 0.5% glutaraldehyde for 10 minutes and washed twice with phosphate buffered saline (PBS)/1 mM $MgCl_2$. Stained cells were examined by light microscopy and photographed.

Preparation of adenovirus/polylysine complex

Polylysine was cross-linked to replication deficient, recombinant adenovirus using a slightly modified version of a procedure described elsewhere (Fisher and Wilson, 1994). In order to malemide-activate the virus, AdRSVlacZ was prepared and concentrated on a CsCl gradient. After centrifugation, this virus was immediately desalted on a 15 ml Sephadex G-50 column equilibrated with PBS, pH 7.0. The viral particle concentration was determined by absorbance at 260 nM, on the assumption that one absorbance unit equals 1 x 10^{12} viral particles per ml. The titered virus was diluted to approximately 5 x 10^{12} particles/ml and sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (Pierce) was added to a final concentration of 25 mM. This reaction was rocked gently at room temperature for 7.5 minutes then stopped by adding 1/10 volume of 1 M Tris, pH 7.0.

While malemide-activating the adenovirus, a thiol group was added onto the amino terminus of poly-L-lysine (54 kDa, Sigma) using 2-iminothiolane essentially as described (Fisher and Wilson, 1994). The 2-iminothiolane was removed on a 15 ml Sephadex G-25 column equilibrated with 100 mM Tris (pH 7.1 at 4°C), 100 mM NaCl, and 2mM EDTA. The first fraction that contained virus as determined by absorbance at 220 nM was saved for further use.

The purified, thiolated polylysine was diluted to an equal volume as the malemide-activated adenovirus using 100 mM Tris (pH 7.1 at 4°C), 100 mM NaCl, and 2 mM EDTA. Then the malemide-activated virus and modified polylysine were mixed and rocked gently at 4°C for 90 minutes. The polylysine is cross-linked to the virus at this step. Unreacted malemide groups were blocked by adding 1/10 volume of 1 M 2-mercaptoethylamine and incubating for 20 minutes at room

temperature. To remove unincorporated polylysine and concentrate the virus, the polylysine modified virus was then centrifuged on a CsCl gradient. The virus was desalted on a 15 ml Sephadex G-50 column. Storage was in 50 mM Tris (pH 7.1), 50 mM NaCl, 1mM EDTA, and 50% glycerol at -80°C. This modified virus will be referred to as AdRSVlaclys.

Successful crosslinking was verified by a gel mobility shift assay. In a total volume of 100 µl PBS, 200 µg of the plasmid pK7GFP (a gift from Ian Macara, Carey et al., 1996) was incubated with varying amounts of modified or unmodified virus for 30 minutes at room temperature. These samples were resolved by gel electrophoresis on a 1.5% agarose/TAE gel.

Transfection of exogenous DNA in vitro using the adenovirus/polylysine complex

We determined that the amount of cross-linked adenovirus necessary to infect greater than 80% of Hela cells was approximately 1 x 10^5 viral particles per cell. Therefore, this titer was used for the following infections. AdRSVlacZ or AdRSVlaclys was incubated with or without pK7GFP in a total volume of 100 µl PBS for 20-30 minutes at room temperature. Hela cells at 1 x 10^5 cells per well in 12-well dishes were washed once with PBS and 0.5 ml DMEM (2% FBS, 1% P/S) was added to each well. Virus/DNA mixtures were added to their respective wells and the cells were incubated at 37°C overnight. The next day, the medium was replaced with 1 ml DMEM (10% FBS, 1% P/S) per well. Each experiment was performed in duplicate. One set was trypsinized, fixed, and stained with X-gal to monitor infection efficiency. The other set was trypsinized, washed once with PBS, and visualized for GFP using a fluorescence microscope to monitor adenovirus-mediated transfection efficiency. The percentage of infected or

transfected cells was determined with a hemacytometer and data represents the mean ± standard deviation of two experiments.

In vitro limited replication of adenovirus using the adenovirus/polylysine complex

AdRSVlacZ or AdRSVlaclys was incubated with either 2 μg of pUC19 or 2 μg of pE1 in a total volume of 200 μl PBS for 20-30 minutes at room temperature. Hela or SKNSH cells at 1.5 x 10^5 per well in 6-well dishes were washed once with PBS and 1 ml DMEM (2% FBS, 1% P/S) was added to each well. Virus/DNA mixtures were added to their respective wells and the cells were placed at 37°C overnight. The next day, medium was replaced with 2 ml DMEM (2% FBS, 1% P/S). Cells were fed at days 3 and 6 with 2 ml DMEM (2% FBS, 1% P/S). No medium was removed during this time. On day eight post infection, the cells were fixed and stained with X-gal to monitor limited replication of the adenovirus. Staining was either done in the dish and subsequently photographed or done in suspension and percent of cells infected was determined with a hemacytometer.

In vivo limited replication of adenovirus

Nude mice (CD-1 *nu/nu*, Charles River) were injected i.p. with 1 x 10^7 Hela cells. Four days later the mice were injected with a virus/DNA mixture in 100 μl of PBS using a Hamilton syringe. Each injection contained 7 x 10^9 particles of AdRSVlaclys with either 2 μg of pUC19 or 2 μg pE1. Two mice were injected with PBS only. These injections were repeated every other day for a total of four injections.

Eight days after the final injection (to allow time for limited replication) mice were sacrificed. The peritoneal cavity was washed twice with 1.5 ml PBS and the resulting cell suspension was removed and saved. These samples were centrifuged

at 350 x *g* in a microcentrifuge for two minutes, washed once with PBS, and recentrifuged. The resulting pellets of Hela and blood cells were resuspended in 1 ml DMEM (10% FBS, 1% P/S) each and placed in 6-well dishes in a total volume of 2 ml medium. These dishes were cultured at 37°C for two days in order to allow most of the blood cells to die or lyse. Then the dishes were washed 6 times with PBS and the remaining Hela cells were fixed and stained with X-gal for 48 hours at 37°C. Cells were examined by light microscopy and photographed. Percentage of infected cells was determined for each sample. Groups were compared using a one-sided student t test.

Detection of the adenovirus hexon protein

Twenty-four hours after infection with the chemically modified AdRSVlaclys and 2 μg of either pUC12 or pE1, Hela cells were harvested and attached to microscope slides. Immunohistochemistry using a FITC-conjugated anti-hexon IgG (Chemicon, Temecula, CA) was done using the protocol recommended by the supplier. Microscopy was done using a Zeiss fluorescent microscope.

RESULTS

Independent co-delivery of the pE1 plasmid and adenovirus in vitro

In order to test whether exogenous E1 plasmid DNA could transcomplement replication deficient adenovirus *in vitro*, an independent plasmid lipofection was followed by AdRSVlacZ infection on Hela or SKOV3 cells. A low titer of AdRSVlacZ was used for these experiments in order to make viral replication easily detectable. Over the course of the next 6 to 8 days, cells initially transfected with pE1 showed a substantially greater proportion of infected cells than those initially transfected with the pUC19 control (Fig. 2A). The staining pattern on E1 transfected cells was present in clusters, indicating areas where limited replication and subsequent cell lysis occurred. There was a greater amplification of the adenovirus in the Hela cells. This may be due to either greater amplification of virus in Hela cells or greater susceptibility to adenovirus infection of Hela cells. This verifies our hypothesis that co-delivery of pE1 will support viral replication.

Although this shows that the co-delivery concept is feasible, the methodology used for these pilot experiments is not practical for general *in vivo* use. Liposomes have been shown to deliver genes *in vivo*, but the efficiency varies dramatically depending on cell type/animal model and would probably need to be extensively optimized for most scenarios (Gao and Huang, 1995). Thus, other methods were explored for the co-delivery of pE1.

Co-delivery of plasmid DNA with an adenovirus/polylysine complex

Polylysine modified adenovirus has been shown to complex with and transfect plasmid DNA (Wagner et al., 1992; Cristiano et al., 1993; Fisher and Wilson, 1994). We planned to use such a virus to co-deliver the pE1 plasmid. Our

method of crosslinking polylysine to AdRSVlacZ was based on the procedure of Fisher and Wilson. This previously reported method essentially modified the entirety of the adenoviral capsid. The modified virus, AdRSVlaclys, would bind to plasmid DNA (Fig. 3).

Next we aimed to demonstrate that AdRSVlaclys could transfect plasmid DNA without the assistance of tertiary substrates (such as a polylysine modified cellular ligand). This would simplify infection procedures for future *in vivo* experiments. Approximately 15% of Hela cells infected with AdRSVlaclys also introduced the pK7GFP plasmid when examined by fluorescence microscopy (Fig. 4). Because fluorescence microscopy to detect GFP is not terribly sensitive, we expect that the actual percentage of transfected cells is higher. In contrast, cells exposed to plasmid alone or plasmid with AdRSVlacZ exhibited no detectable GFP expression. Thus, the polylysine attached to adenovirus allows co-delivery of plasmid DNA.

Based on these experiments, we predicted that delivery of the pE1 plasmid complexed with AdRSVlaclys would result in significantly enhanced gene transfer due to a round of viral replication. Transfection of only 15% of infected cells translates into a great deal of additional virus when one considers that the typical adenovirus life cycle results in 10^4 viral progeny (Shenk, 1996). When AdRSVlaclys and pE1 were used to infect Hela cells, a large increase of infected cells was observed by X-gal staining when compared to the control AdRSVlaclys/pUC19 sample (Fig. 5). Once again, clusters of blue cells indicated areas of viral replication. As expected, infection of Hela cells with AdRSVlacZ resulted in the same amount of gene transfer regardless of preincubation with pE1

or pUC19. This experiment generated analogous results when repeated with SKNSH (human neuroblastoma) cells, indicating that this effect is not specific to Hela cells (Fig. 6).

It is possible that the replication observed in AdRSVlaclys/pE1 infected/transfected cells was due to recombination of the replication defective AdRSVlaclys genome with the transfected pE1 plasmid to generate replication competent helper virus. This presents a potential safety concern for future *in vivo* applications of this method. To address this concern, the AdRSVlaclys/pE1 infection was repeated on Hela cells. These infections were harvested at four days post-infection, freeze-thawed, and the supernatant of this lysate was used to infect a new monolayer of Hela cells in a 35 mm dish. These cells were monitored over the next 14 days for the appearance of cytopathic effect (CPE). As a positive control, Hela cells were incubated with various dilutions of sub360, which is both the original adenovirus strain used to generate AdRSVlacZ (Davidson et al., 1994) and the predicted recombination product of pE1 and AdRSVlaclys. Dilutions ranged from 10^4 viral particles (the amount of virions generated from one viral life cycle) to 10^8 viral particles per well. All dilutions of sub360 produced CPE within the two week time period, as evidenced by completely detached, swelled or lysed cells. Monolayers from Hela cells cultured with the AdRSVlaclys/pE1 infection extract were intact after 14 days and showed no signs of CPE (data not shown). This strongly suggests that the limited viral replication observed with AdRSVlaclys/pE1 infections is due to transcomplementation, not recombination. This also suggests that helper virus is not a significant contributing factor to any of the results presented in this study.

Enhanced gene transfer in vivo using limited replication of AdRSVlaclys

In order to demonstrate the ability of limited replication to improve transduction efficiency *in vivo*, AdRSVlaclys complexes were injected into mice bearing Hela ascites tumors. After a series of four injections, the virus was allowed eight days to replicate, infect more cells, and express β-galactosidase. At the time of sacrifice, there was no apparent toxicity to mice injected with virus. X-gal staining revealed a low percentage of infected Hela cells in mice injected with PBS or AdRSVlaclys/pUC19 (data summarized in Table 1). Higher infection percentage was apparent in Hela cells isolated from mice injected with AdRSVlaclys/pE1 when compared to the PBS group ($p < 0.05$) or the AdRSVlaclys/pUC19 group ($p < 0.01$). Transduction efficiency reached close to 90% in one of the mice (Fig. 7C). *In vivo*/virus replication was confirmed. E-1 transfected cells infected with the Lac Z recombinant adenovirus, but not the PUC-19-transfected cells infected with the same recombinant virus, produced the adenovirus late hexon protein (Fig. 8). This data provides evidence that limited replication results in a significantly higher proportion of tumor cells infected *in vivo*.

DISCUSSION

Viral vectors are extremely attractive as gene transfer vehicles due to their evolved mechanisms for introducing genetic material into cells. This has led to extensive efforts in adapting viruses for the delivery of therapeutic genes (Miller, 1992). In some cases (such as cystic fibrosis), delivery of the therapeutic gene theoretically only needs to take place in a small proportion of affected cells in order to produce significant benefits. However, in other cases, infection of the majority of the target cells may be necessary. For example, the proliferative nature of cancer dictates that it is necessary to infect the majority, if not all, transformed cells in order to achieve true clinical benefit from gene transfer. This problem is exacerbated by the extreme difficulty of transducing the majority of targeted cells *in vivo*, since viruses can only diffuse for limited distances in solid tissues.

Limited viral replication represents a strategy for improving the efficiency of transducing target cells *in vivo*. The feasibility of limited replication has been demonstrated here *in vitro* with several cell lines, using a model system consisting of an E1-deleted adenoviral vector that could introduce exogenous E1 sequences (on a plasmid), permitting replication. Because these DNA sequences were separate from the adenovirus genome and thus were not incorporated into adenoviral progeny, all of the resultant virions were replication defective, ending replication after one round. Minimal helper virus was produced which indicates that this method may be as safe as traditional adenovirus administration. We have also extended this study to show that limited replication of adenovirus leads to enhanced gene transfer *in vivo*. The potential of the E1 plasmid with the adenovirus vector to form a replication competent adenovirus could be further

minimized by using an E1 plasmid with no sequences that are shared with the adenovirus vector.

The model system presented in this study was only to demonstrate the principle of limited replication and can obviously be improved. Safety concerns regarding the transforming potential of integrated pE1 plasmid sequences can be alleviated by engineering a suicide gene such as the herpes simplex virus *thymidine kinase* gene into the plasmid. Cells that contain the pE1 plasmid can then be purged from the body using gancyclovir after the course of treatment. Furthermore, adenovirus crosslinking procedures that result in greater transfection and infection activity have been reported, and would likely lead to better *in vivo* results (Cristiano et al., 1993). Ideally though, chemical modification of the adenovirus capsid would not take place because it invariably decreases infectability. A plausible alternative would have the E1 sequences packaged inside the adenovirus capsid (but not in the genome), which would result in 100% of infected cells to undergo a round of replication, provided that the cells are permissive. Current technology does not allow us to do this. Another possible scenario would be placing the E1 sequences in the adenovirus genome, but under tumor specific promoters. This could be difficult since it is likely that leaky E1 expression would occur due to viral enhancers present throughout the adenovirus genome (Shenk, 1996).

Limited replication could be especially useful for cancers arising in cavities where adenovirus is easily administered. Two examples of this are bladder and ovarian cancer. Bladder cancers are superficial tumors which arise focally or diffusely, initially penetrating only a few cell layers of the bladder luminal epithelium. Ovarian cancer initially spreads in the peritoneal cavity. In both of

these cases, it may be possible to infect all transformed cells using limited replication of adenovirus. This would also be a probable improvement upon current gene transfer methods in solid tumor masses, although it is still unlikely that all the cells of a large tumor mass can be transduced. In summary, we have shown that limited replication of adenovirus is safe and results in enhanced gene transfer both *in vitro* and *in vivo*. This addresses the problem of inadequate gene transfer in cancer gene therapy and may eventually lead to potential clinical benefits in the future.

ACKNOWLEDGMENTS

We thank Jennifer Sanderson for expert secretarial assistance. This manuscript is dedicated in the memory of Happy and Bunny Han. This work was supported by The Department of Defense (941407), and the National Institutes of Health (1P01CA75136-01A1).

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Table 1. Efficiency of gene transfer by *in vivo* limited replication.

mouse number	*treatment*	*percent Hela cells blue*
1	AdRSVlaclys/pUC19	14.0
2	AdRSVlaclys/pUC19	23.0
3	AdRSVlaclys/pUC19	9.4
4	PBS	6.5
5	PBS	3.5
6	AdRSVlaclys/pUC19	15.3
7	AdRSVlaclys/pE1	21.3
8	AdRSVlaclys/pE1	66.4
9	AdRSVlaclys/pE1	84.6
10	AdRSVlaclys/pE1	69.6

Fig. 1. Schematic diagram of limited replication *in vivo*. On the left, conventional techniques result in gene transfer only near the surface of tissue. On the right, limited replication allows virus diffusion to deeper areas in the tissue. Viral replication is occuring in dark cells.

Fig. 2. Lipofection of plasmid DNA followed by adenoviral infection. Cells were lipofected with 1 μg of pUC19 (left) or pE1 DNA (right), then infected with AdRSVlacZ. X-gal staining is shown at six and eight days. A. Hela cells at six days. B. Hela cells at eight days. C. SKOV3 cells at six days. D. SKOV3 cells at eight days.

Fig. 3. Gel mobility shift of polylysine modified adenovirus. AdRSVlaclys, AdRSVlacZ, or no virus was incubated with 200 μg of plasmid DNA. Attachment of DNA to AdRSVlaclys results in a large complex with neutralized charge that does not run into the gel. a. 2.5 x 10^9 particles AdRSVlaclys + DNA. b. 5 x 10^9 particles AdRSVlaclys + DNA. c. 1 x 10^{10} particles AdRSVlaclys + DNA. d. 2.5 x 10^{10} particles AdRSBlaclys + DNA. e. 2.5 x 10^{10} particles AdRSVlacZ + DNA. f. 2.5 x 10^{10} particles AdRSVlacZ (no DNA). g. DNA only.

Fig. 4. Transfection of plasmid DNA with AdRSVlaclys. AdRSVlaclys, AdRSVlacZ, or no virus was incubated with pK7GFP before infection of Hela cells. A. AdRSVlaclys infected cells under light. B. AdRSVlaclys infected cells using fluorescence microscopy. C. The infection and transfection efficiencies of various combinations of virus and DNA on Hela cells. Magnification 200X. Addition of plasmid DNA caused a slight decrease in infectivity. This is most likely due to a reduced cell membrane binding because of the negatively charged DNA.

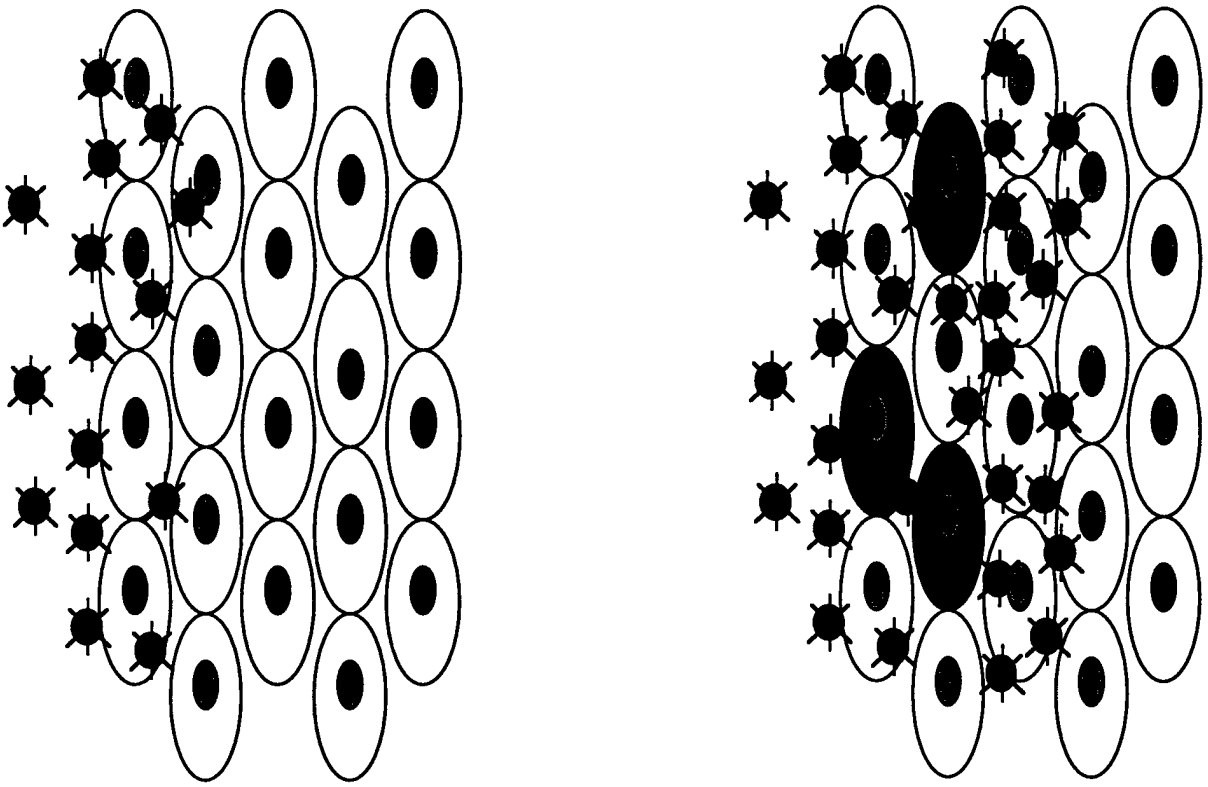
Fig 5. Limited replication of AdRSVlaclys in Hela cells. A. AdRSVlacZ preincubated with pUC19. B. AdRSVlacZ preincubated with pE1. C. AdRSVlaclys preincubated with pUC19. D. AdRSVlaclys preincubated with pE1. Magnification 200X. Percent infected ± s.d. is indicated in the corner of each photograph.

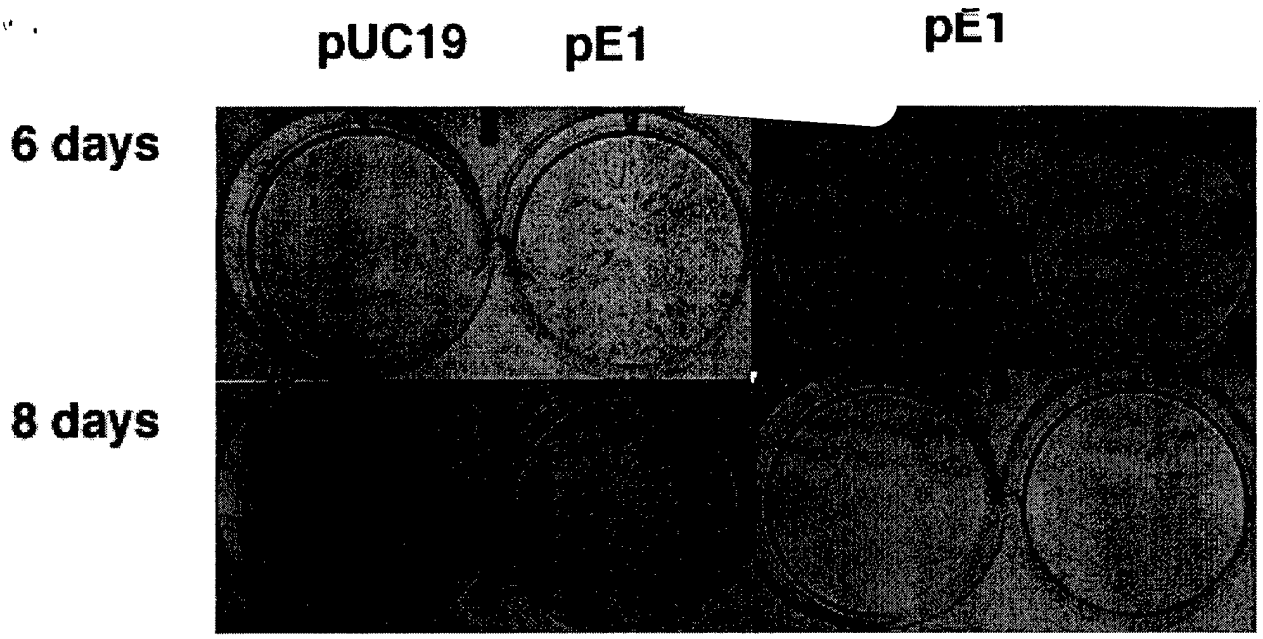
Fig. 6. Limited replication of AdRSVlaclys in SKNSH cells. The experiment described in Figure 5 was extended to SKNSH cells.

Fig. 7. Examples of limited replication of AdRSVlaclys *in vivo*. Ascites were injected, harvested, and stained as described in Materials and Methods. A. PBS (mouse #5). B. AdRSVlaclys with pUC19 (mouse #6). C. AdRSVlaclys with pE1 (mouse #9). Magnification 200X.

Fig. 8. *In vivo* expression of the adenovirus hexon protein in Hela cells. Immunohistochemistry showing expression of the adenovirus hexon protein in Hela cells infected with AdRSVlaclys conjugated with either pUC19 (A) or pE1 (B). Magnification 200x.

Figure 1





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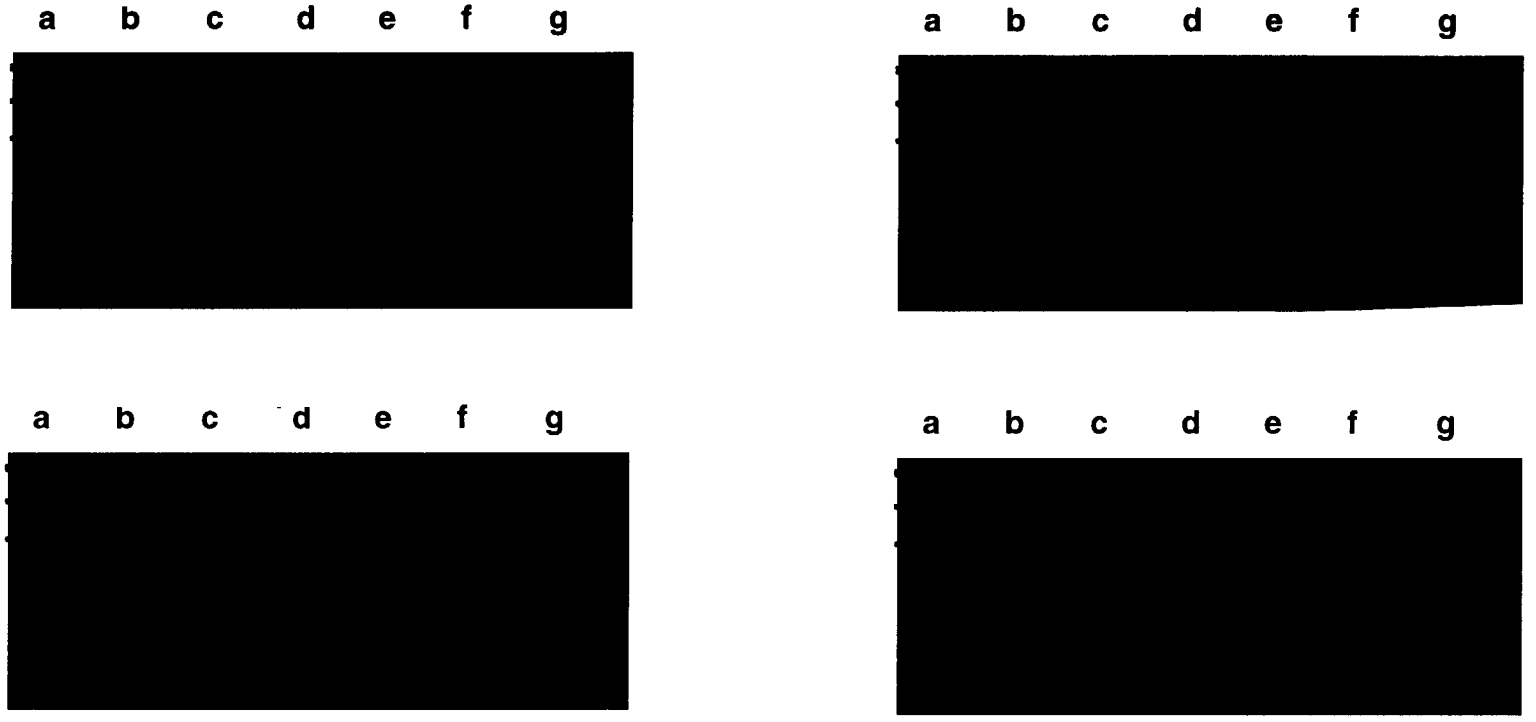
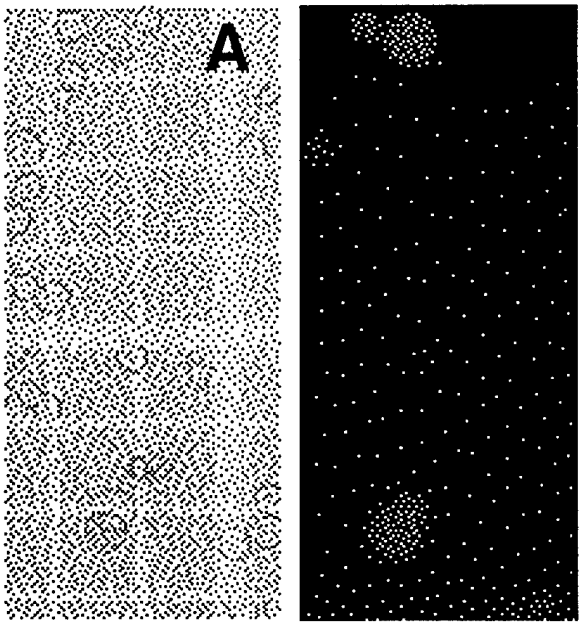


Figure 3

Figure 4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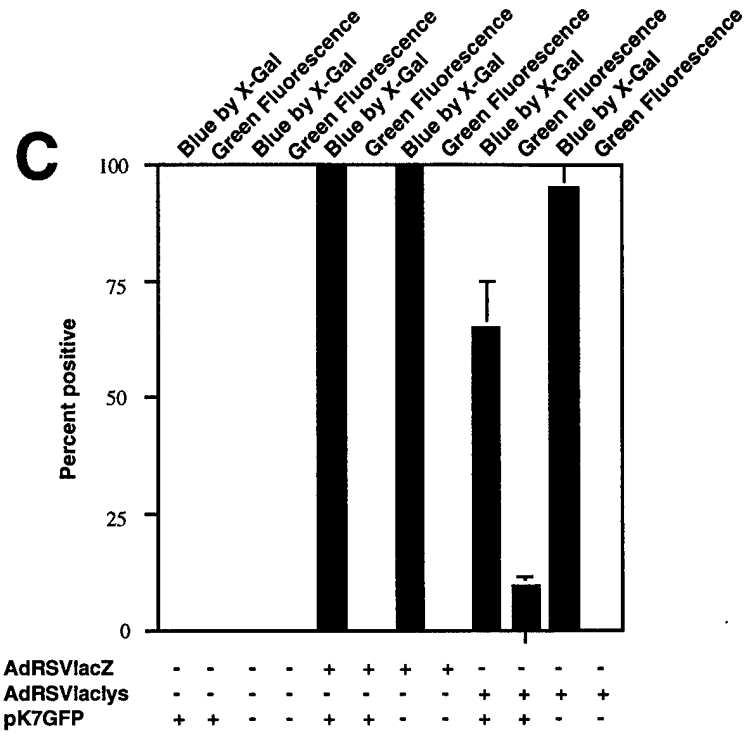
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Green Fluorescence
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75
50
25
0
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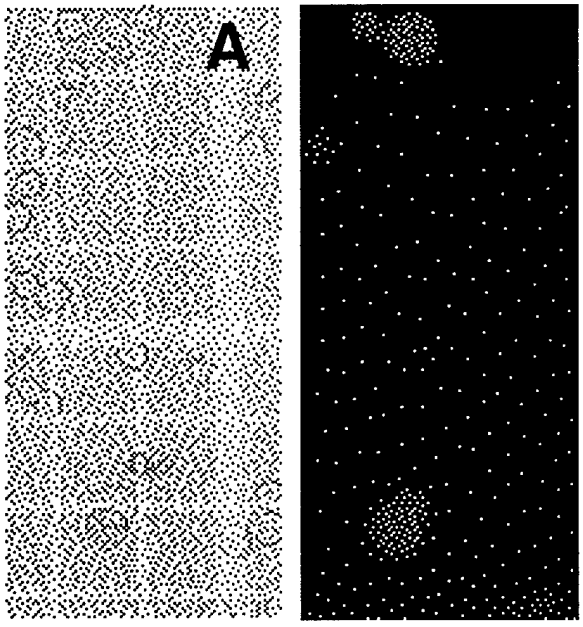
AdRSVlaclys
pK7GFP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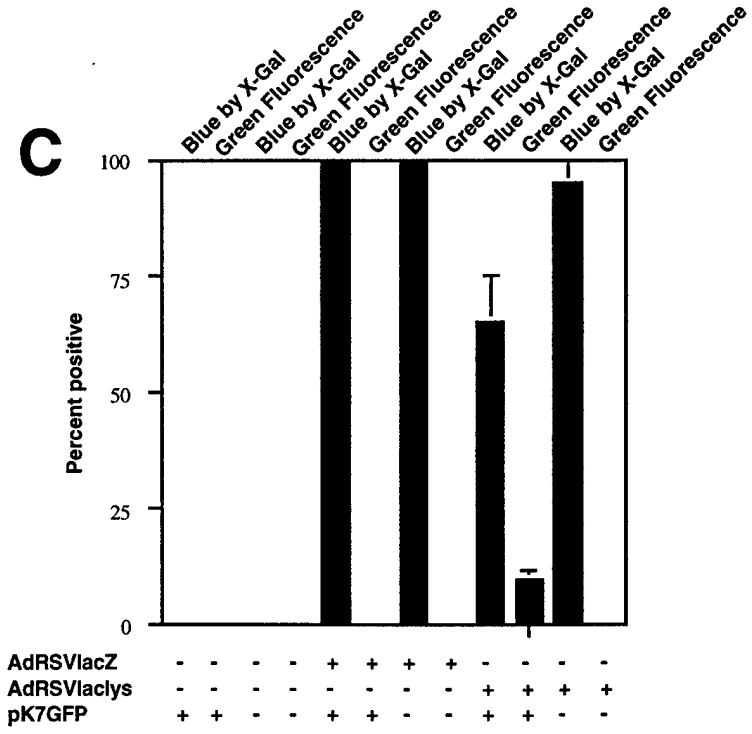
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Percent positive
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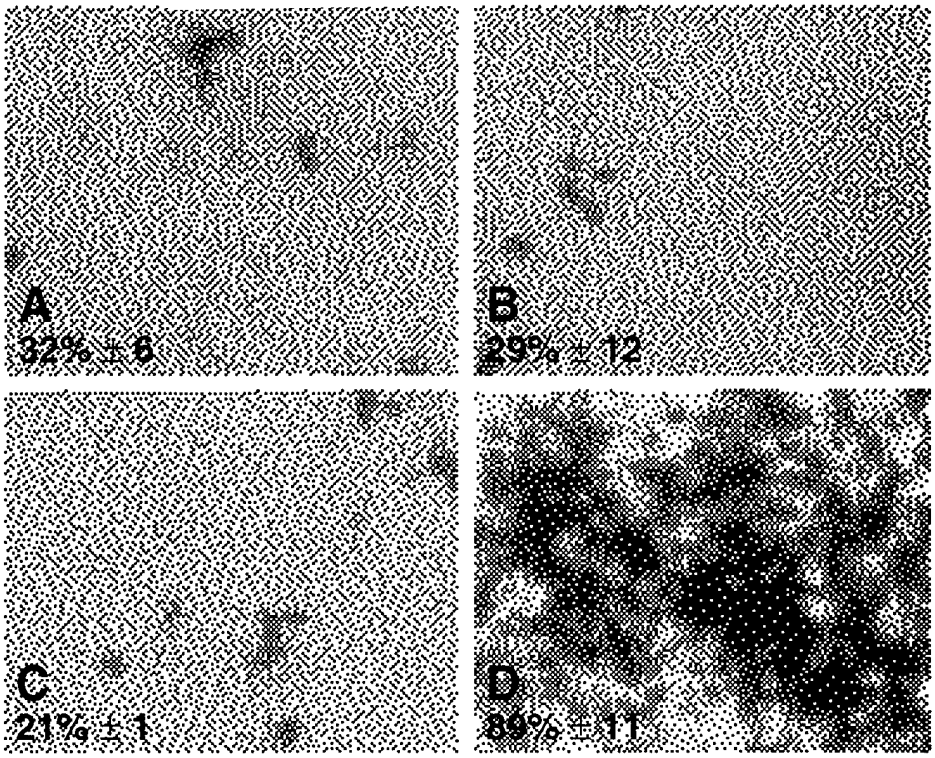
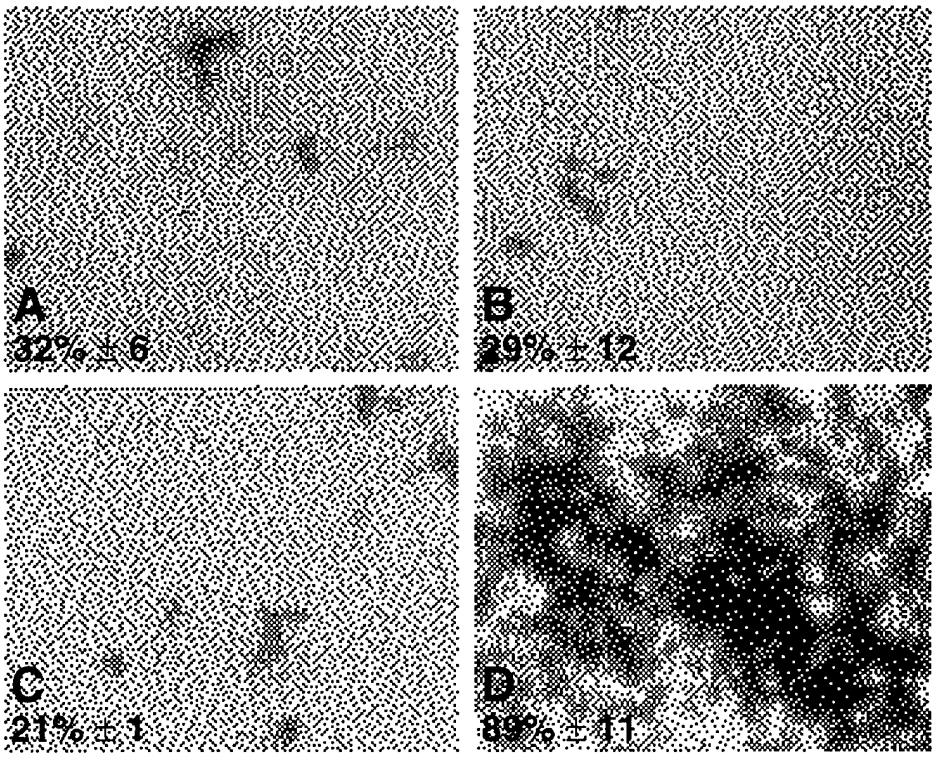
pK7GFP

Figure 5





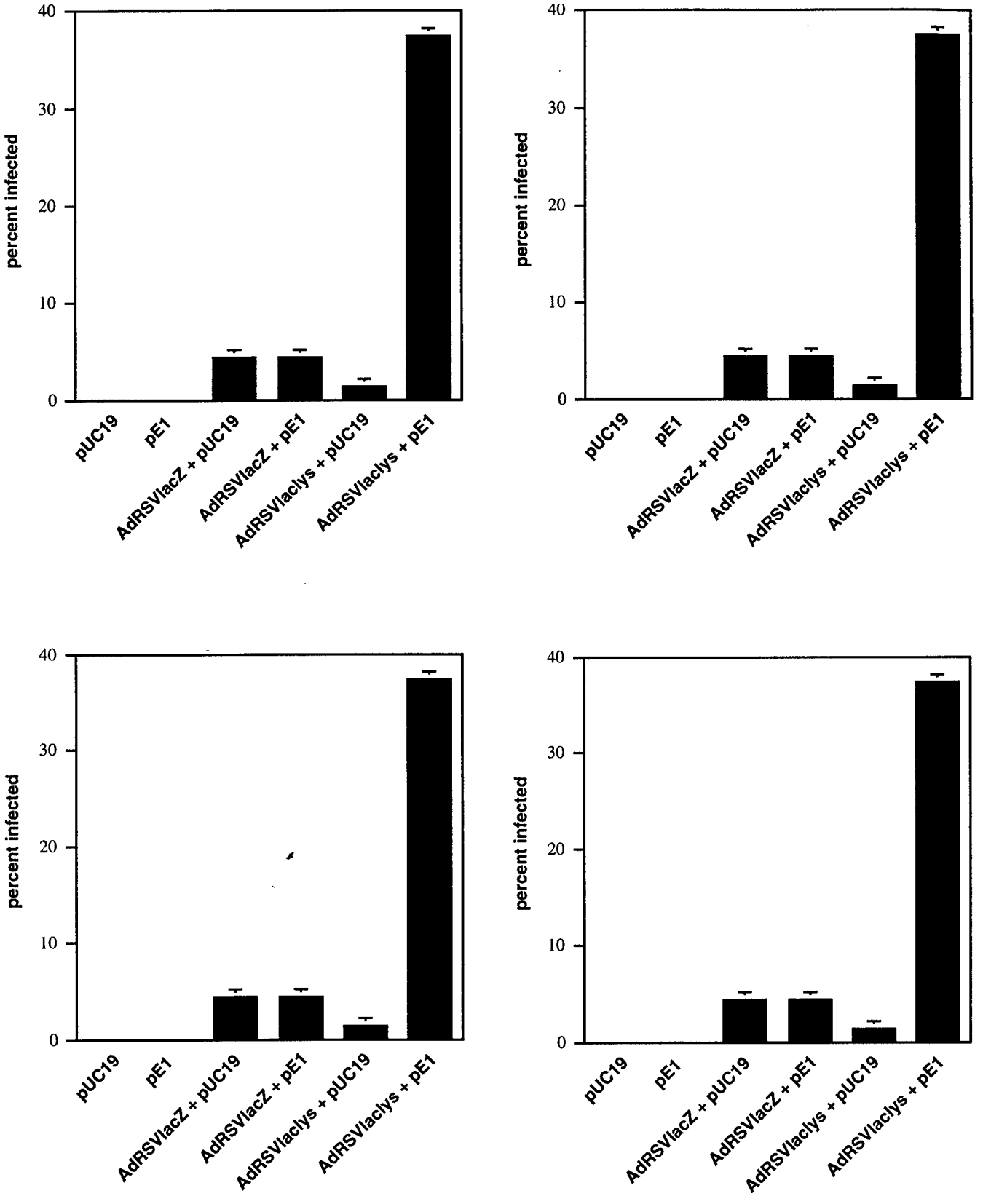
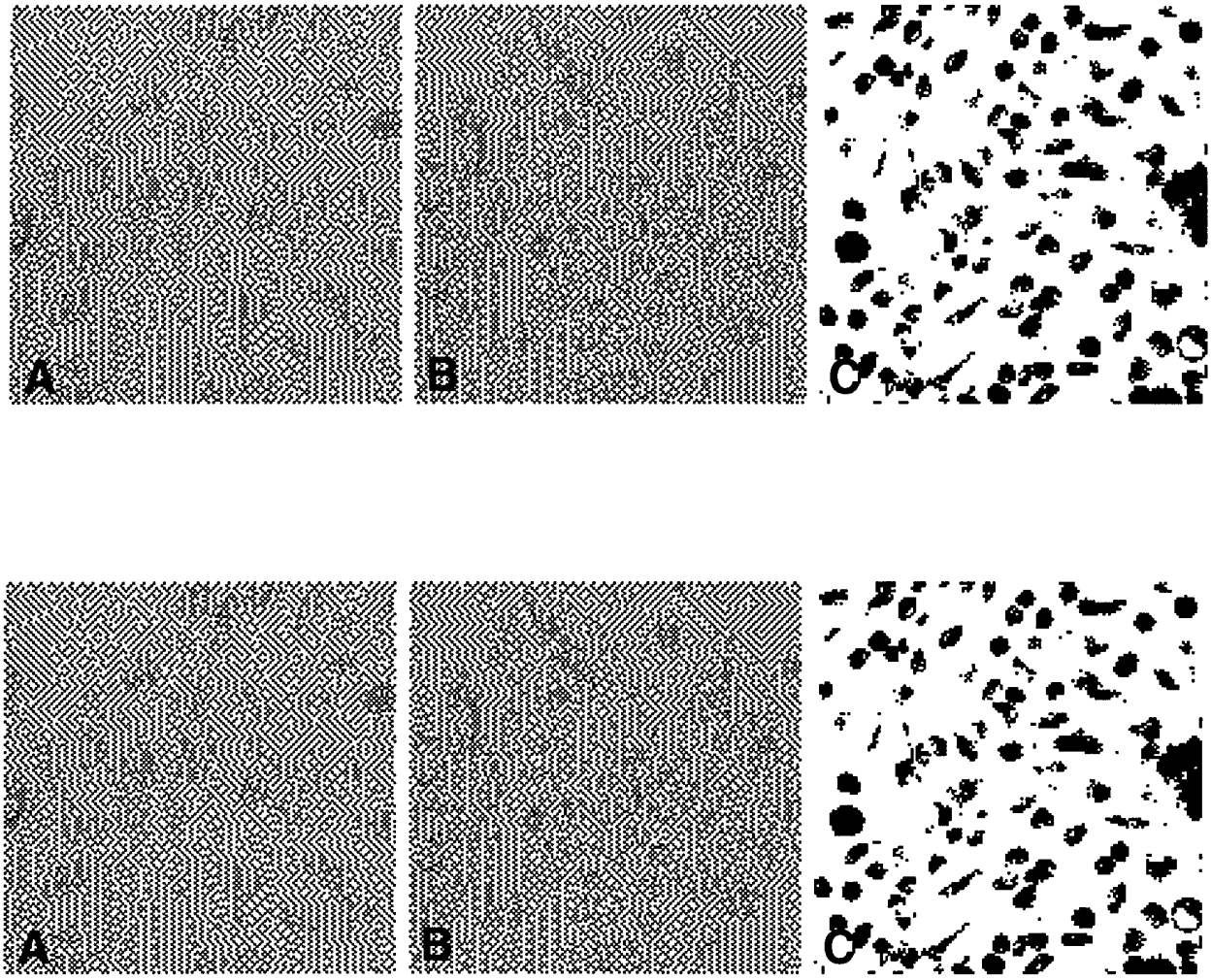

40
30
20
10
0
percent infected
pUC19
pE1
AdRSVlacZ + pUC19
AdRSVlacZ + pE1
AdRSVlaclys + pUC19
AdRSVlaclys + pE1

Figure 6

Figure 7



Carcinogenesis vol.16 no.8 pp.1761–1767, 1995

Bcl-2 protects murine erythroleukemia cells from p53-dependent and -independent radiation-induced cell death

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To better understand the molecular basis of radiation-induced cell death, we studied the role of the *bcl-2* oncogene and the *p53* tumor suppressor gene in this process. A temperature-sensitive mutant of murine p53 ($p53^{Val-135}$) and/or *bcl-2* was transfected into murine erythroleukemia cells (MEL, DP16-1, which are null in p53). We demonstrate that radiation-induced cell death occurs by both p53-dependent and -independent pathways and overexpression of *bcl-2* modulates both pathways. When viability was measured 24 h post-radiation, cells that had been briefly exposed to wtp53 immediately after X-ray irradiation had decreased survival as compared to unirradiated cells expressing wtp53 or X-ray irradiated DP16-1 cells. However, at later times X-ray irradiated parental DP16-1 cells also had decreased survival compared to the unirradiated control. This decrease in survival began 48 h following radiation. Bcl-2 prevented radiation-induced cell death in DP16-1 cells expressing wtp53 and delayed radiation-induced cell death in DP16-1 cells without wtp53. X-ray irradiated cells expressing wtp53 displayed microscopic and biochemical characteristics consistent with cell death due to apoptosis. DP16-1 cells which were untransfected or co-transfected with wtp53 and *bcl-2* displayed characteristics of cells undergoing necrosis. These results suggest that radiation-induced cell death occurs by both p53-dependent and p53-independent pathways. The p53-dependent pathway results in cell death via apoptosis and occurs approximately 24 h following radiation. The p53-independent pathway does not appear to involve apoptosis and occurs at a later time, starting 48 h after X-ray exposure. Thus, bcl-2 protects cells from p53-dependent radiation-induced apoptotic cell death and attenuates p53-independent radiation-induced cell death.

Introduction

Ionizing radiation induces cell death by two distinct mechanisms, necrosis and apoptosis (1,2). Necrosis is the form of cell death associated with massive tissue damage which in individual cells is frequently preceded by increased plasma membrane permeability, swelling of the matrix of mitochondria, and irreversible cell swelling and lysis (2,3). Exposure of thymocytes to high doses of radiation (more than 10 Gy) has been demonstrated to produce cell necrosis (1). An alternative form of cell death termed apoptosis results from a programmed sequence of events. This form of death is characterized by condensation of chromatin, alterations in membrane permeability, and degradation of nuclear DNA into oligonucleosomal fragments (3). Apoptosis can be induced by XRT and other DNA damaging agents (3). In irradiated thymocytes, the onset of apoptosis is rapid with DNA degradation and loss of membrane permeability accompanied by uptake of vital dyes occurring within 6–7 h following irradiation (4–6).

p53 Has roles in the cellular responses to DNA damage induced by XRT (7,8). The *p53* tumor suppressor gene is one of the most commonly mutated genes found in human malignancies (9) and has been shown to play an important role in transformation. In addition to a role in the genesis of cancers, the modulation of apoptosis has been shown to play a role in cancer treatment. In addition to having a role in regulating cellular apoptosis (10–12), wild-type p53 (wtp53) has been postulated to play a role in DNA repair following XRT (13) by arresting irradiated cells in the G1 phase of the cell cycle (14). Levels of p53 protein increase following treatment with XRT (8). Therefore, the DNA repair capabilities following XRT may be altered by expression of a nonfunctional mutant p53. In thymocytes, wtp53 has been shown to be necessary for apoptosis induced by XRT but not Ca^{2+} ionophore or dexamethasone (14). Other data support the fact that cells without wtp53 have increased radioresistance (15). Consistent with the hypothesis that p53 has a role in radiation-induced cell death is the observation that cells from individuals with Li-Fraumeni syndrome that carry a germ-line p53 mutation exhibit increased resistance to XRT (16). Elegant studies with p53 'knockout' mice showed that X-ray irradiation and certain chemotherapeutic agents kill cells through p53-dependent apoptosis (14,17).

In contrast to p53, overexpression of the *bcl-2* (B-cell lymphoma/leukemia 2) gene confirms cellular resistance to apoptosis (18,19). The *bcl-2* oncogene was initially discovered by its involvement in the t(14;18) chromosomal translocation found in non-Hodgkin's lymphomas (20). The Bcl-2 protein is localized to the outer mitochondrial membrane, RER and nuclear envelope and is thought to contribute to oncogenesis by suppressing signals that induce apoptotic cell death (21). For example, overexpression of Bcl-2 can prevent hematopoietic and neural cell apoptosis induced by growth factor withdrawal (18,19), and Bcl-2 also prevents or delays apoptosis induced by irradiation, glucocorticoids, heat shock and multiple chemotherapy agents (22,23). Although *bcl-2* alone does not stimulate cell proliferation or cause transformation, it cooperates with c-*myc* (13,20,24) and members of the *ras* family (13) to transform. Taken together, these results suggest that apoptosis is a critical defense mechanism against malignant transformation. Because Bcl-2 production is a common feature of many carcinomas, lymphomas and leukemias, it is thought that bcl-2 may play a role in the resistance of therapy. Indeed, expression of *bcl-2* in leukemia, carcinoma of the prostate and neuroblastoma has been shown to be a marker for poor prognosis (25–28).

Although it is known that wtp53 induces apoptosis (10–12) and cooperates with XRT to enhance cell death (13,14) and that Bcl-2 protects from some forms of apoptosis (21,22,24) and partially protects from XRT-induced cell death, it is not

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known if elevated levels of Bcl-2 will block p53-dependent apoptotic death or p53-independent radiation-induced cell death. Such knowledge is important because many tumors which require radiation therapy (e.g. breast cancer, prostate cancer) often express high levels of Bcl-2 (25–28). Therefore, to better understand the role of p53 and Bcl-2 in XRT-induced cell death, we introduced either a temperature-sensitive mutant of p53 (29) and/or *bcl-2* into DP16-1, a murine erythroleukemia (MEL) cell line which has no endogenous expression of p53 protein (30) and low levels of endogenous Bcl-2 (31). Previous data from our laboratory show that continuous long exposure to wtp53 is associated with loss of viability which occurs over 48 h (12). In this report, we demonstrate that XRT-induced cell death occurs by both p53-dependent and p53-independent pathways. Analysis of genomic DNA isolated from irradiated cells suggests that death in cells expressing wtp53 occurs via apoptosis. Additionally, cell death also occurs via a p53-independent pathway that occurs at a later time and appears to be via necrosis. Overexpression of Bcl-2 modulates both cell death pathways: it protects cells from p53-dependent radiation-induced apoptosis and it attenuates p53-independent radiation-induced cell death.

Materials and methods

Cell culture and DNA transfections

DP16-1 MEL cells were grown in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% calf serum, 2 mM glutamine, 100 U of penicillin per ml, and 100 μg of amphotericin B per ml. DNA transfections were performed by electroporation as previously described (32). Transfection mixes consisted of 10^7 cells in transfection buffer (272 mM sucrose, 7 mM phosphate [pH 7.4], 1 mM $MgCl_2$) with either 10 μg of *Bam*HI digested p53^{ts}neo or 10 μg of *Bam*HI digested p53^{ts}hygro with 10 μg *Sal*I digested pSFFVbcl-2neo (21). pSV-2 neo (33) was used for control transfections. Transfected cells were selected for the appropriate resistance gene in medium containing 1 mg of geneticin (G418, Gibco) per ml or 1200 mg per ml of hygromycin B (Calbiochem).

The construction of p53^{ts}neo has been previously described (12). This plasmid contains a genomic clone for p53Val-135 (29) under control of the RSV LTR promoter and a neomycin resistance gene. P53^{ts}hygro was derived from p53^{ts}neo by deletion of the *Bam*HI/*Hin*dIII fragment containing the neomycin resistance gene and insertion of a 2.3kb *Bam*HI/*Hin*dIII fragment containing the hygromycin resistance gene from p65hygro (34). Single cell clones were isolated by limiting cell dilution in 96-well microtiter plates. These cell lines have been previously described in detail (12,31).

Radiation

Cells were X-ray irradiated using a Cobalt-60 X-ray machine (dose rate: 120 cGy/min). Cells were given various doses of X-rays, up to 800 cGy, and were either returned to 37.5°C or placed at 32.5°C for 8 h to express wtp53. Viability was determined at various time points following radiation by trypan blue exclusion. All experiments were performed at least three times.

Clonogenic assay

Cells treated with varying doses of X-ray irradiation or unirradiated control cells were placed at 32.5°C for 8 h to express wtp53. Two hundred cells were plated in methylcellulose and allowed to grow at 37.5°C for 10 days to determine colony forming ability.

DNA analysis

Genomic DNA was prepared as previously described (35). Briefly, cells were suspended in a buffer consisting of 20 mM Tris (pH 7.5), 10 mM EDTA, and 300 mM NaCl, lysed by adding sodium dodecyl sulfate (SDS) to 0.5%, digested with proteinase K and then subjected to organic extraction and ethanol precipitation. DNA samples (3 μg) were analyzed by electrophoresis through a 1.5% agarose gel containing 0.5 μg of ethidium bromide per ml.

Microscopy

For Hoechst 33342 staining, 5×10^4 cells were washed with PBS and then collected onto microscope slides using a cytospin centrifuge. The cells were fixed with ice cold methanol for 20 min and then washed at 4°C with PBS. The cells were then incubated for 10 min at room temperature in PBS with 5 μM Hoechst 33342. The slides were washed twice with PBS, after which a drop of Slowfade Antifading Reagent was added. Cells were viewed by fluorescent microscopy.

For acridine orange/ethidium bromide (Sigma) staining, 2×10^5 cells were washed twice with PBS. Cells were collected by centrifugation and resuspended in PBS with 3.3 ng/ml of acridine orange and ethidium bromide. Cells were immediately viewed by fluorescent microscopy.

Results

p53 Enhances X-ray irradiation lethality

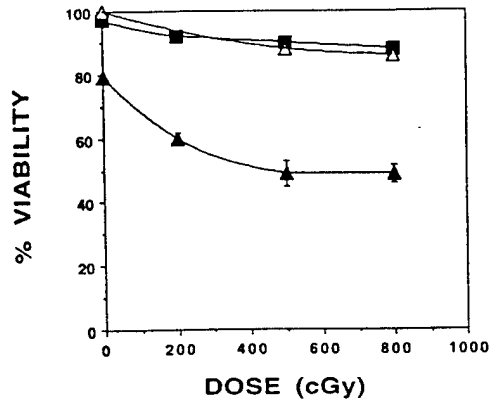
In order to investigate the role of p53 in radiation-induced cell death of MEL cells we utilized the MEL cell line, DP16-1. These cells, which do not express endogenous p53 (30), were transfected with *p53*^{ts}hygro (a plasmid which expresses p53^{val135}, a temperature-sensitive mutant of murine p53, and a hygromycin resistance gene). The p53^{val135} protein encoded by *p53*^{ts}hygro is in the mutant conformation when cells are grown at 37.5°C, but assumes a wild-type conformation when MEL cells are grown at 32.5°C (29). Previous data from our laboratory showed that while transfected cells grew normally in the presence of mutant p53 (37.5°C), expression of wtp53 (32.5°C) was associated with growth arrest and a loss of viability which occurred over a 48–120 h period (12). This cell death is restricted to cells that express wtp53 during a critical period in the G1 phase of the cell cycle (12). In randomly growing cells, only a small percentage of cells are in this part of the cell cycle at any given time and are killed due to wtp53 exposure. Therefore, a brief exposure of wtp53 was chosen to determine the relationship between p53 expression and radiation-induced cell death. Exposure to wtp53 (32.5°C) for 1, 2, 4 and 8 h prior to XRT and immediately after XRT for 4 and 8 h was performed. The most significant effect of XRT was seen following an 8 h exposure to wtp53 immediately after XRT (data not shown); therefore, this exposure time was chosen to study the interactions of p53 with XRT in MEL cells. An 8 h induction of wtp53 in unirradiated cells had a minimal effect on viability (80 ± 0.7% viability at 24 h).

DP16-1 cells transfected with *p53*^{ts}hygro were exposed to wtp53 by temperature downshift for 8 h following varying doses (0, 200, 500 and 800 cGy) of XRT. XRT had very little effect on the short term viability of the parental DP16-1 cells at 24 h, as measured by trypan blue exclusion, even up to doses of 800 cGy (Figure 1A). In contrast, the irradiated cells expressing wtp53 showed a decline in viability at 24 h after irradiation compared to unirradiated cells expressing wtp53 (Figure 1A). There was little or no additional effect, however, of increasing dose of XRT from 200 cGy to 800 cGy on cell viability (Figure 1A). The dosage of 200 cGy totally abolished the colony forming ability in these cells (Figure 1B). XRT of 800 cGy had little effect on cell viability (Figure 1A) in the presence of mutant p53 (37.5°C).

p53-Independent X-ray death occurs later and is less efficient than p53-dependent cell death

wtp53 Expression is required for normal thymocytes to undergo cell death following XRT (14). To determine if XRT-induced lethality can also occur independent of p53 expression, DP16-1 MEL cells, which are null in p53, were treated with varying doses of XRT (0, 200, 500 and 800 cGy). Short term viability was determined 24 and 48 h after XRT. X-ray irradiated DP16-1 cells had decreased survival compared to the unirradiated control (Figure 2A). This decrease in survival was most significant at 48 h following 800 cGy radiation. There was a decline in viability with increasing doses of XRT, but this was only seen at 48 h (Figure 2A). There was only a slight to

A

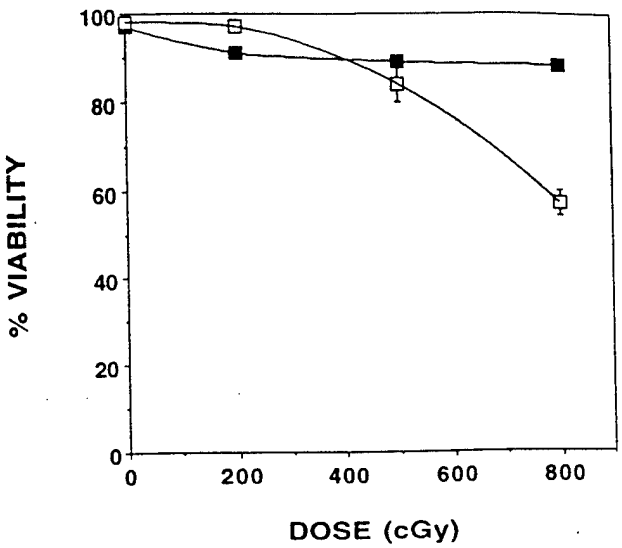


B

Dose	0 cGy	200 cGy	500 cGy	800 cGy
No. Colonies	46 ± 2.0	1.0 ± 0.7	0 ± 0	0 ± 0

Fig. 1. Effect of wtp53 expression on XRT-induced cell death. (A) At 0 h, logarithmically growing cells transfected with p53 were X-ray irradiated with 0, 200, 500, or 800 cGy and transferred from 37.5°C to 32.5°C for 8 h (wtp53). DP16-1 MEL cells and transfected MEL cells (mutant p53) were also irradiated and kept at 37.5°C. Symbols: ■, DP16-1 X-ray irradiated; ▲, wtp53 X-ray irradiated; △, mutant p53 X-ray irradiated. Viability was determined by trypan blue staining at 24 h following radiation. The percentage viability was determined by taking the ratio of viable cells to total cells counted. Note the rapid loss of viability of the cells expressing wtp53. (B) Clonogenic assay. Cells transfected with p53 were X-ray irradiated with 0, 200, 500 and 800 cGy and transferred from 37.5°C to 32.5°C for 8 h. Two hundred cells were plated in methylcellulose and allowed to grow for 10 days and number of colonies were determined. There was a decrease in colony forming ability in the X-ray irradiated cells, however, there was no radiation dose effect.

A



B

Dose	0 cGy	200 cGy	500 cGy	800 cGy
No. Colonies	111± 1.0	96 ± 16	4 ± 0.7	0.5 ± 0.7

Fig. 2. p53-Independent XRT-induced cell death. (A) p53-independent XRT-induced cell death occurs later and is less efficient. DP16-1 MEL cells were X-ray irradiated with 0, 200, 500, or 800 cGy and viability was determined by trypan blue staining at 24 and 48 h following XRT. Symbols: ■, DP16-1 X-ray irradiated (24 h); □, DP16-1 X-ray irradiated (48 h). Decreased survival of X-ray irradiated DP16-1 cells was noted and occurred at 48 h following XRT. Dose effect was seen at 48 h following XRT. (B) Clonogenic assay. DP16-1 cells were X-ray irradiated with 0, 200, 500 and 800 cGy and placed at 37.5°C for 8 h. Two hundred cells were plated in methylcellulose and allowed to grow for 10 days and number of colonies were determined. There was a decrease in colony forming ability following 800 cGy of radiation but little effect at 200 or 500 cGy.

modest decline in viability of cells that received 200 cGy or 500 cGy of XRT, respectively (Figure 2A). Complete loss of colony formation was also not seen until the cells had been exposed to 800 cGy of XRT (Figure 2B).

Bcl-2 protects against p53-dependent, and delays p53-independent radiation-induced lethality

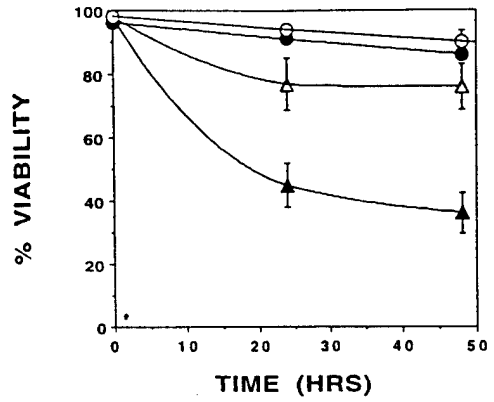
Previous data from our laboratory have demonstrated that in contrast to cells expressing only p53, cells expressing both p53 and *bcl-2* had prolonged survival at 32.5°C (31). Bcl-2 delayed but did not prevent p53-induced apoptosis (31). To determine whether Bcl-2 protects against XRT-induced cell death through a p53-dependent or -independent pathway, we analyzed DP16-1 cells, $p53^{ts}$hygro-transfected DP16-1 cells, and $p53^{ts}$hygro and/or pSFFV *bcl-2* neo (a plasmid which expresses human Bcl-2 and geneticin resistance genes) -transfected DP16-1 cells. Parental DP16-1 cells and DP16-1 cells expressing wtp53 and Bcl-2, either alone or together, were irradiated with 800 cGy. DP16-1 cells expressing Bcl-2 and p53 were X-ray irradiated with 800 cGy and shifted to 32.5°C for 8 h to express wtp53. In comparison to irradiated cells expressing only wtp53, irradiated cells expressing both wtp53 and *bcl-2* had prolonged survival (Figure 3A). Bcl-2 and p53 co-transfected cells had 90.0 ± 1.0% viability at 24 h compared to 45.0 ± 7.0% for cells transfected with p53 alone. Colony assay showed *bcl-2* protection against radiation-induced clonogenic death in the presence of wtp53 (compare Figure 3B with Figure 1B).

In order to determine if *bcl-2* could protect against p53-independent XRT-induced cell death, DP16-1 cells transfected only with *bcl-2* were X-ray irradiated with 800 cGy. At 48 h, DP16-1 cells transfected with *bcl-2* had increased viability over untransfected DP16-1 cells (Figure 4A). Bcl-2 transfected cells had 81.0 ± 1.0% viability at 48 h compared to 49.0 ± 3.5% for untransfected cells. Despite the improved viability at 48 h following XRT, colony assay revealed that Bcl-2 did not significantly improve colony forming ability of the irradiated parental cells (Figure 4B).

Mechanism of p53-dependent and -independent radiation-induced cell death

P53 enhances XRT-induced lethality in MEL cells. This cell death is most pronounced at 24 h following radiation. To determine whether the loss of viability was through the process of apoptosis, genomic DNA was isolated from DP16-1 cells expressing wtp53 24 h following 800 cGy of XRT. DNA from apoptotic cells has a characteristic fragmentation pattern secondary to internucleosomal cleavage (36). Approximately 20% of the unirradiated DP16-1 cells that expressed p53 for a brief period underwent apoptosis (Figure 1A) and there was

A

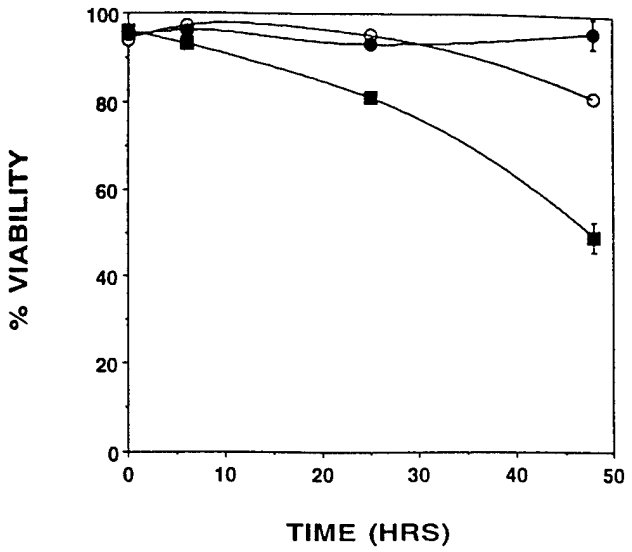


B

Dose	0 cGy	200 cGy	500 cGy	800 cGy
No. Colonies	206 ± 2.1	81.0 ± 3.5	11.5 ± 4.9	4.0 ± 2.8

Fig. 3. Bcl-2 protects cells against p53-dependent XRT-induced cell death. (**A**) Logarithmically growing DP16-1 cells transfected with *bcl-2* and p53 were X-ray irradiated with 800 cGy and transferred from 37.5°C to 32.5°C for 8 h. Symbols: ▲, p53 X-ray irradiated at 32.5°C; ○, p53-Bcl-2 X-ray irradiated at 32.5°C; ●, p53-Bcl-2 unirradiated at 32.5°C: △, p53 unirradiated at 32.5°C. Viability was determined by trypan blue staining. Bcl-2 protects cells from p53 and XRT-induced lethality. (**B**) Clonogenic assay. DP16-1 cells transfected with both *bcl-2* and p53 were X-ray irradiated with 0, 200, 500 and 800 cGy and placed at 32.5°C for 8 h. Two hundred cells were plated in methylcellulose and allowed to grow for 10 days and number of colonies were determined. Colonies were formed following 200, 500, or 800 cGy of radiation. Bcl-2 protects wtp53 cells from XRT-induced clonogenic death.

A



B

Dose	0 cGy	200 cGy	500 cGy	800 cGy
No. Colonies	72 ± 10	63 ± 12	20 ± 5	0.5 ± 0.7

Fig. 4. Bcl-2 delays p53-independent XRT-induced cell lethality. (**A**) At 0 h, logarithmically growing DP16-1 cells transfected with Bcl-2 were X-ray irradiated with 800 cGy. Symbols: ■, DP16-1 X-ray irradiated; ●, Bcl-2 unirradiated; ○, Bcl-2 X-ray irradiated. Viability was determined by trypan blue staining. There was increased viability of Bcl-2 cells compared to X-ray irradiated DP16-1 cells. (**B**) Clonogenic assay. DP16-1 cells transfected with *bcl-2* were X-ray irradiated with 0, 200, 500 and 800 cGy and placed at 37.5°C for 8 h. Two hundred cells were plated in methylcellulose and allowed to grow for 10 days and number of colonies were determined. There was a decrease in colony forming ability following 500 and 800 cGy of radiation but little effect at 200 cGy.

a faint apoptotic ladder seen in DNA extracted from these cells. A much larger amount of DNA from X-ray irradiated cells expressing wtp53 was degraded into the characteristic nucleosomal ladder of cells undergoing apoptosis (Figure 5A). This shows that wtp53 cooperates with X-ray irradiation to induce apoptosis. In contrast only high molecular weight DNA is detected from unirradiated cells without wtp53. The ability of Bcl-2 to block apoptosis was demonstrated by the lack of DNA fragmentation in cells expressing both p53 and *bcl-2* following 800 cGy of XRT.

Figure 2 demonstrates that XRT lethality also occurs by a p53-independent pathway. To determine if this death occurs via apoptosis, genomic DNA was isolated from DP16-1 cells 24 and 48 h following 800 cGy of XRT. The characteristic DNA fragmentation pattern for apoptosis was not seen in either the unirradiated control or X-ray irradiated cells at 24 h (Figure 5A) and 48 h (data not shown). Fluorescent light microscopy demonstrates that within 8 h after exposure to XRT, cells that express wtp53 begin to undergo changes characteristic of apoptosis, including shrunken morphology and condensation of chromatin and nuclear fragmentation. In contrast, irradiated cells that do not express wtp53, develop nuclear and cytoplasmic swelling, characteristic of cells undergoing necrosis (Figure 5B). This finding reveals that XRT-induced cell death can occur by a necrosis pathway which is independent of p53.

Discussion

In this study we have shown that in MEL cells, like normal thymocytes, wtp53 is required for radiation-induced apoptosis. We have also demonstrated that p53-independent radiation-induced cell death is most likely due to necrosis. Bcl-2 appears to protect cells from both forms of radiation lethality. There is evidence that p53 plays an important role in many of the cell's responses to XRT. p53 Expression increases following XRT (8) and is obligate for the transient G1 arrest seen in normal cells following DNA damage caused by agents including XRT (7,13). Furthermore thymocytes from p53 null mice are resistant to XRT-induced killing (14). This has led to the proposal that p53 may serve as an important checkpoint in the cell cycle prior to entering S phase. In hematopoietic cell lines, p53 kills cells in G1 but not in G0 or S phase (12). However, the cell cycle pattern of p53 killing in response to XRT has not been determined. Following XRT, G1 arrest occurs and levels of p53 increase which may allow for synergistic cell death secondary to apoptosis. Our data confirm the observation that wtp53 increases sensitivity of cells to XRT and that in MEL cells it is essential for XRT-induced apoptosis.

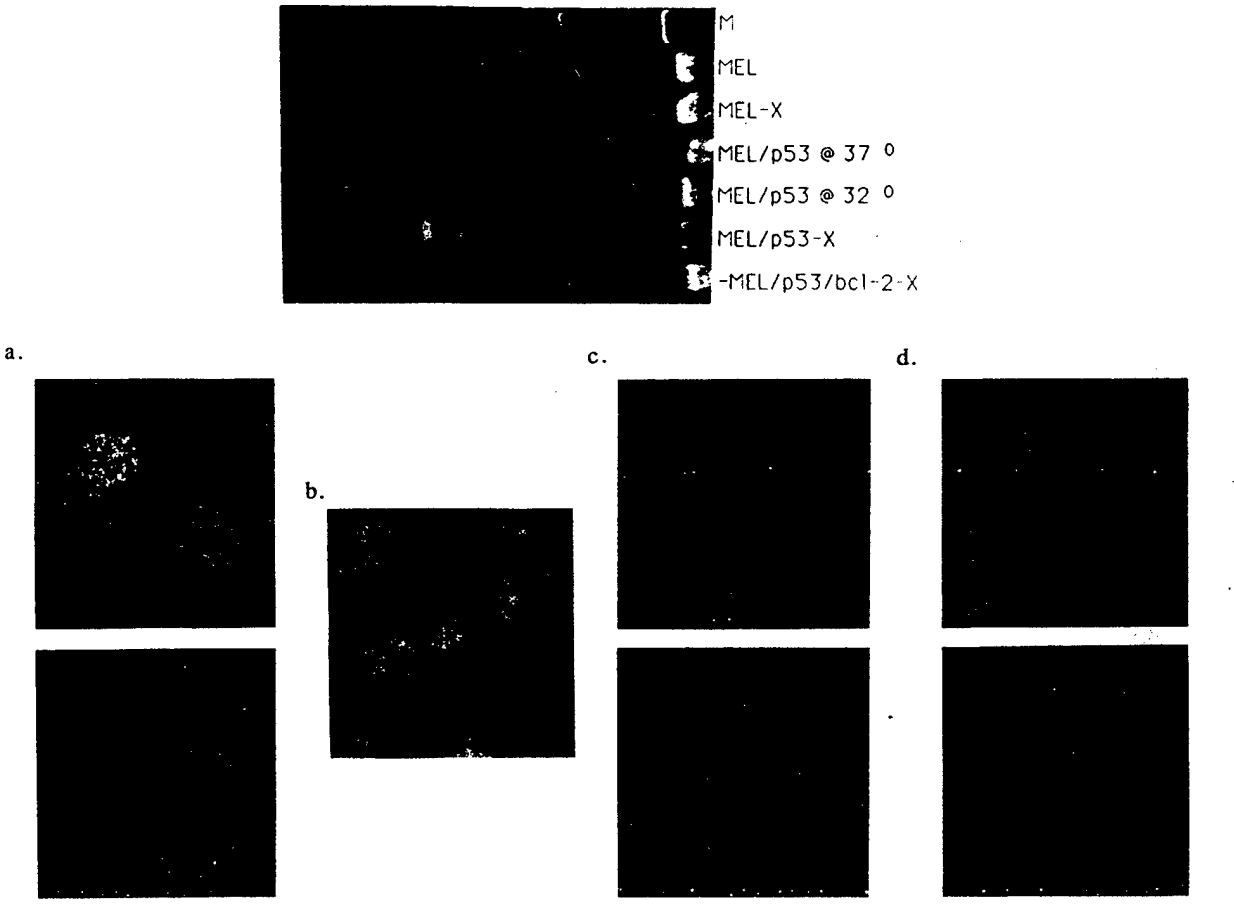


Fig. 5. Light microscopy and DNA fragmentation in p53-dependent XRT-induced apoptosis. **(A)** Cells were X-ray irradiated with 800 cGy and genomic DNAs (3 μg) were isolated 24 h following radiation. DNA was extracted from unirradiated DP16-1 cells **(lane b)**, X-ray irradiated DP16-1 cells **(lane c)**, unirradiated p53 transfected cells incubated at 37.5°C for 24 h **(lane d)**, unirradiated p53 transfected cells incubated at 32.5°C for 24 h **(lane e)**, X-ray irradiated p53 transfected cells at 32.5°C **(lane f)**, and X-ray irradiated p53 and Bcl-2 co-transfected cells after incubation at 32.5°C for 24 h following XRT **(lane g)** then analyzed by electrophoresis through a 1.5% agarose gel. DNA fragmentation consistent with apoptosis was seen in the p53 transfected cells at 32.5°C but not in the X-ray irradiated DP16-1 cells at both 24 h (lane c) or 48 h (data not shown) or co-transfected p53 and Bcl-2 cells (lane g). **(B)** Cells were irradiated, stained with the indicated fluorescent dye and analyzed by light microscopy (1000 ×). Panel a, eight hours after irradiation, $p53^{neo}$ (top) or parental (bottom) MEL cells were stained with ethidium bromide and acridine orange. Parental (top) or $p53^{neo}$ (bottom) MEL cells were stained with Hoechst 33342. Unirradiated $p53^{neo}$ cells (panel b), and $p53^{neo}$ (top) and parental (bottom) cells 8 h (panel c), or 24 h (panel d) after irradiation are shown. Note that irradiated cells expressing wtp53 demonstrate nuclear condensation, fragmenting and cytoplasmic blotting while irradiated parental cells demonstrate nuclear and cytoplasmic swelling.

Radiation is felt to induce cell death by damaging DNA and this can result in inhibition of DNA synthesis and/or replication by G1 and G2 arrest (8). XRT also results in the generation of free radicals which are felt to induce oxidative base damage to DNA including DNA strand breaks and the production of thymine glycols (1). Cells exposed to radiation die via both apoptosis and necrosis. At high doses of XRT cells display features typical of necrosis whereas after low doses of XRT, cells may undergo apoptotic death (1,37). Our data show that in MEL cells apoptotic death is dependent on wtp53 whereas necrotic cell death is independent of wtp53 expression. Our data also indicate that p53-independent cell death (necrosis) occurs later and less efficiently than p53-dependent XRT-induced apoptosis (Figures 1, 2). p53-Dependent radiation-induced cell death requires lesser amounts of radiation than p53-independent radiation-induced cell death. In MEL cells expressing wtp53, there is a maximal response at 200 cGy, doses above which there is minimal additional killing. The p53-independent XRT-induced cell death pathway, however, requires greater amounts of radiation with minimal amount of death occuring after the exposure to 200 cGy, and maximum killing seen with dosages of 800 cGy. This suggests that the apoptotic p53-dependent pathway requires a lesser amount of radiation to achieve efficient cell killing with little additional dose effect. Thus, the p53-independent pathway is less efficient, requires higher doses of XRT, and is XRT dose-dependent.

Tumors of epithelial origin often contain mutations which result in loss of the *p53* genes (38). However, a certain percentage of such tumors express wtp53, suggesting that there are other means to escape p53 functions. Such genetic differences may be relevant to response to treatment and our data support the hypothesis that expression of wtp53 can confer sensitivity to ionizing radiation (13,14). In contrast with p53, Bcl-2 has been shown to inhibit apoptotic death in many systems (18,19). Bcl-2 is also known to protect some cells from radiation-induced apoptotic death but the p53 status is not known. A recent report showed radiation could induce

apoptosis by a p53-independent mechanism and this was inhibitable by *bcl-2* (39). In contrast, our studies show that p53-independent radiation death was most likely due to necrosis and not due to apoptosis and that *bcl-2* delays radiation-induced necrosis. This suggests that in cells which co-express p53 and *bcl-2*, any process which down-regulates Bcl-2 may trigger or facilitate apoptosis.

We have now shown that Bcl-2 completely protected MEL cells from p53-dependent radiation death and attenuated p53-independent radiation-induced death. We predict that tumors that express high levels of wtp53 and low levels of Bcl-2 would be most sensitive to DNA damaging agents such as XRT. Tumor cells expressing wtp53 that would normally undergo efficient XRT-induced apoptotic cell death would be protected by Bcl-2. Although Bcl-2 is thought to regulate apoptosis, recently it has been shown that it might also protect normal cells from necrosis secondary to glutathione deprivation (40). Thus, tumors with high levels of Bcl-2 would be expected to be more resistant to XRT pathways. Indeed, it is possible that expression of Bcl-2, or a homolog such as Bcl-x_L (41), might be one of the major factors which predict response to radiation therapy.

In this paper we have demonstrated that wtp53 is essential for XRT-induced apoptosis. We have also shown that an alternative form of XRT-induced cell death occurs which is independent of p53 and is most likely necrotic death. Bcl-2 appears to modulate both forms of XRT lethality. Bcl-2 prevents p53-dependent XRT-induced cell death and attenuates p53-independent radiation-induced cell death. It is becoming apparent that deregulated expression of Bcl-2 often occurs in many epithelial tumors including cancer of the prostate and breast, as well as leukemia, lymphomas, and pediatric neuroblastoma (25–28). These observations suggest that strategies designed to down-regulate or functionally inhibit Bcl-2 might markedly increase the effectiveness of radiation therapy.

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Received on December 23, 1994; revised on April 26, 1995; accepted on May 5, 1995

Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 11024–11028, November 1995
Medical Sciences

A recombinant *bcl-x$_S$* adenovirus selectively induces apoptosis in cancer cells but not in normal bone marrow cells

(*bcl-2*/gene therapy/stem cells)

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Communicated by J. L. Oncley, University of Michigan, Ann Arbor, MI, July 20, 1995 (received for review June 2, 1995)

ABSTRACT **Many cancers overexpress a member of the *bcl-2* family of inhibitors of apoptosis. To determine the role of these proteins in maintaining cancer cell viability, an adenovirus vector that expresses *bcl-x$_S$*, a functional inhibitor of these proteins, was constructed. Even in the absence of an exogenous apoptotic signal such as x-irradiation, this virus specifically and efficiently kills carcinoma cells arising from multiple organs including breast, colon, stomach, and neuroblasts. In contrast, normal hematopoietic progenitor cells and primitive cells capable of repopulating severe combined immunodeficient mice were refractory to killing by the *bcl-x$_S$* adenovirus. These results suggest that Bcl-2 family members are required for survival of cancer cells derived from solid tissues. The *bcl-x$_S$* adenovirus vector may prove useful in killing cancer cells contaminating the bone marrow of patients undergoing autologous bone marrow transplantation.**

It is becoming increasingly apparent that disruption of the pathways regulating programmed cell death (PCD; apoptosis) is integral to the etiology of a variety of cancers. Expression of certain tumor-suppressor proteins such as p53 can induce some cancer cells to undergo apoptosis (1–4). Oncogenes have also been implicated in PCD. For example, several groups have observed that deregulated expression of c-*myc* can activate the apoptosis pathway (5–7). Furthermore, *bcl-2*, the gene deregulated in most follicular lymphomas, primarily functions to inhibit apoptosis (for reviews, see refs. 8 and 9). *bcl-2* encodes an intracellular membrane-associated protein that has been localized to the mitochondria, endoplasmic reticulum, and perinuclear regions (9). Although expression of *bcl-2* does not stimulate cell proliferation, it can cooperate with c-*myc* (10, 11) to cause transformation. Moreover, expression of high levels of Bcl-2 protein in normal or neoplastic cells delays or inhibits PCD induced by many factors including p53, Myc, chemotherapy, and ionizing radiation (8, 9). A large percentage of epithelial and hematopoietic tumors overexpress Bcl-2 (8, 9). Furthermore, overexpression of Bcl-2 is correlated with poor prognosis and resistance to treatment in patients with neuroblastoma (12), prostatic cancer (13), and some forms of leukemia (14).

Recently, a homolog of *bcl-2*, called *bcl-x*, has been identified and partially characterized (15, 16). As a result of alternative splicing, two *bcl-x* mRNA species, designated *bcl-x$_L$* and *bcl-x$_S$*, were identified in the human. The former, like *bcl-2*, inhibits apoptosis (15, 17). The latter is thought to function as a repressor of Bcl-2, as it enhances apoptotic signals in cells that express Bcl-2 (15). Postulating that inactivation of Bcl-2 or Bcl-x_L might increase the susceptibility of cancer cells to PCD, an adenovirus vector that expresses Bcl-x_S protein was constructed. Primary carcinoma cells, as well as cell lines derived from solid tumors, rapidly underwent cell death after infection with the *bcl-x$_S$* adenovirus. In primary breast cancer cells and multiple breast cancer cell lines, expression of *bcl-x$_S$* was associated with rapid induction of cell death. In contrast, human hematopoietic progenitor cells exposed to this virus maintained viability and retained their ability to reconstitute the bone marrow of irradiated immune-deficient mice. Blocking Bcl-2 or Bcl-x_L function by the *bcl-x$_S$* adenovirus appears to provide another strategy for inducing apoptosis in tumor cells. These findings have important implications for cancer therapy.

MATERIALS AND METHODS

Construction of the *bcl-x$_S$* Adenovirus. The plasmid pBSbcl-x_S (15) was digested with *Eco*RI, and customized *Bam*HI linkers were ligated onto the ends; the ≈536-bp fragment was purified and ligated into *Bam*HI-digested pAd5RSV plasmid (18). The pAd5RSV *bcl-x$_S$* recombinant virus was isolated by *in vivo* homologous recombination between the linearized pAd5RSV *bcl-x$_S$* plasmid and the replication-deficient sub360 adenovirus that has a partial deletion of the E3 region and deletion of the E1A and E1B genes (18). Three recombinant viruses that expressed Bcl-x_S protein in infected cells were plaque-purified twice. Large preparations of adenovirus were made by infecting 293 cells and purifying crude virus preparations by CsCl centrifugation (19).

Cell Infections with Recombinant Adenoviruses. The number of adenovirus particles in viral stocks was determined by spectrophotometry (18). Adenovirus titers were determined by limiting dilution and plaque formation of 293 cells exposed to the virus dilutions. Absence of replication-competent virus was confirmed by limiting dilution and plaque formation of HeLa cells exposed to the virus dilutions. Each cell line was infected with a stock of the β-galactosidase virus of known titer and then stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) to determine the number of viruses per cell needed to infect 85–98% of each cell line. Unless otherwise indicated, the concentration of the *bcl-x$_S$* adenovirus used to infect cells was identical to the number of β-galactosidase viruses that infected 85–98% of the cells. Cells were exposed to the adenovirus vectors for 4 hr in serum-free medium. The medium was then replaced with tissue culture medium/2% fetal calf serum, and the cells were incubated overnight. The next day the medium was removed and replaced with tissue culture medium/10% serum. Cell viability was measured by trypan blue exclusion.

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Abbreviations: PCD, programmed cell death; SCID, severe combined immunodeficiency virus.

Analysis of Bcl-x Protein. Immunoblot analysis of Bcl-x proteins was done as described (17) by using a rabbit anti-Bcl-x antiserum. The blots were developed with epichemiluminescence substrate (Amersham).

Hematopoietic Cell Assays. Bone marrow was harvested from normal human volunteers by using a protocol approved by the University of Michigan Institutional Review Board. Low-density mononuclear cells were isolated using Ficoll/Hypaque centrifugation essentially as described (20). Hematopoietic cells (1×10^6) were mixed with 1.5×10^4 pSV_2-neo-transfected SHSY-5 neuroblastoma cells. The cells were then infected with adenovirus in serum-free medium containing kit ligand at 1 mg/ml and interleukin 3 at 10 mg/ml. After 2 days of culture, cells were harvested, and triplicate progenitor assays using 1×10^4 cells were done as described (20). To assay for viability of SHSY-5 cells that had been mixed with hematopoietic cells and then exposed to the *bcl-x$_S$* adenovirus, cells were grown in tissue culture medium containing the antibiotic Geneticin at 1 mg/ml to kill the normal hematopoietic cells. To determine the ability of *bcl-x$_S$* adenovirus-infected cells to engraft nonobese diabetic (Nod)/severe combined immunodeficiency (SCID) mice, 1×10^7 low-density bone marrow mononuclear cells were infected with 0, 2×10^3, 5×10^3, or 10^4 adenoviruses per cell and inoculated into the tail vein of irradiated (400 cGy) Nod/SCID mice (21). After 1 mo, the mice were sacrificed, and the bone marrow was harvested and analyzed for human hematopoietic cells essentially as described (22).

RESULTS

Construction of Adenoviral Vector Expressing Bcl-x$_S$. We and others have recently demonstrated that the overexpression of Bcl-2 oncogene will block p53-induced apoptosis (9). This finding led to the prediction that inhibition of Bcl-2 function might induce apoptosis in tumor cells that express wild-type p53. To test this hypothesis, an adenovirus vector that expresses *bcl-x$_S$*, a functional inhibitor of Bcl-2, was constructed by inserting the *bcl-x$_S$* coding sequences into the pADRSV vector (Fig. 1*A*). Nine virus plaques were isolated by cotransfecting the pADRSV *bcl-x$_S$* construct with the sub360 adenovirus into 293 human kidney cells (23), and the viruses were amplified. Restriction digests and Southern blots revealed three viruses that contained the *bcl-x$_S$* minigene. Immunoblotting using a rabbit polyclonal antibody raised against the Bcl-x protein revealed that MCF-7 breast cancer cells infected with the *bcl-x$_S$* adenovirus, but not with a control adenovirus that contains a β-galactosidase gene, expressed the ≈21-kDa Bcl-x$_S$ protein (Fig. 1*B*).

The *bcl-x$_S$* Adenovirus Is Lethal to a Broad Range of Cancer Cells. MCF-7 breast cancer cells (which express high levels of wtp53 and Bcl-2) infected with the *bcl-x$_S$* adenovirus, but not MCF-7 cells infected with the control virus, began dying 2 days after infection. When cells were analyzed 6 days after infection, the MCF-7 cells infected with the β-galactosidase adenovirus had grown and formed colonies. In contrast, MCF-7 cells infected with the *bcl-x$_S$* adenovirus became rounded, subsequently detached from the tissue culture plastic, and died (Fig. 2*A*). Dying cells infected with the *bcl-x$_S$* adenovirus morphologically resembled cells undergoing apoptosis. They were shrunken with picnotic nuclei and cytoplasmic blebbing (Fig. 2*A*). Furthermore, DNA degradation, a hallmark of apoptosis, was detected *in situ* in virtually all cancer cells infected with the *bcl-x$_S$* adenovirus but not with the control virus using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) assay (24) (data not shown). Several other types of cancer cells including cells of breast, colon, and neuroblastoma origin were also killed by the *bcl-x$_S$* adenovirus (Table 1). As reported, the adenovirus containing the β-galactosidase gene alone demonstrated various degrees

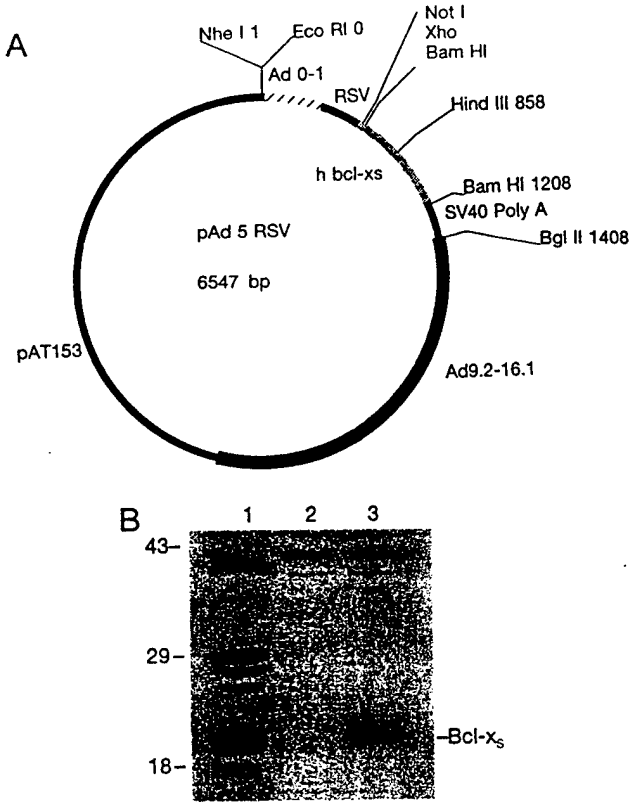

FIG. 1. (*A*) The *bcl-x$_S$* adenovirus. The pRSVAd/*bcl-x$_S$* construct is shown. SV40, simian virus 40; Ad, adenoma virus. (*B*) Immunoblot of lysates from MCF-7 cells. Expression of Bcl-x$_S$ protein was analyzed by SDS/PAGE and immunoblotting with a rabbit polyclonal antiserum (17). Lanes: 1, 200 μg of protein from FL5.12 cells transfected with pSFFV/*bcl-x$_S$* minigene (15); 2, 40 μg of protein from the parental MCF-7 cells infected with β-galactosidase adenovirus; 3, 40 μg of protein from MCF-7 cells infected with *bcl-x$_S$* adenovirus. Molecular size standards are shown at left (in kDa). Note that MCF-7 cells infected with *bcl-x$_S$* adenovirus express the ≈21-kDa Bcl-x$_S$ protein.

of toxicity to some but not all cancer cell lines (25). To determine whether the *bcl-x$_S$* adenovirus can induce cell death in primary cancer cells, breast cancer cells isolated from six patients were exposed to the virus. When infected with β-galactosidase virus at 1×10^3 to 1×10^4 viruses per cell, there was no effect on viability (Fig. 2*B*). In contrast, cells infected with even the lowest concentration of the *bcl-x$_S$* adenovirus showed a marked cytotoxicity (Fig. 2*B*). Primary cells isolated from one of the patients would form colonies in tissue culture. Fifty thousand cells from this patient were cultured after infection with zero or 1×10^4 viruses per cell. Although innumerable colonies formed in the control cultures, none formed in the cells infected with the *bcl-x$_S$* adenovirus (data not shown). The *bcl-x$_S$* adenovirus was also lethal to primary breast cancer cells isolated from five other patients (Table 1).

The ability of *bcl-x$_S$* adenovirus-infected cells to grow *in vivo* was tested. Two days after 5×10^4 RKO colon cancer cells were infected with the *bcl-x$_S$* adenovirus, but not the β-galactosidase virus, the cells began dying. By day 6, only a few of the *bcl-x$_S$* adenovirus-infected cells excluded trypan blue (data not shown). Next, 5 million RKO cells were infected with 2×10^3 *bcl-x$_S$* adenoviruses per cell or β-galactosidase viruses per cell. Uninfected cells or colon cancer cells infected with the control virus formed tumors in 7 out of 10 and 2 out of 5 injected nude mice, respectively (Table 2). In contrast, RKO cells infected with the *bcl-x$_S$* adenovirus did not form tumors in any of the 15 mice injected with such cells (Table 2).

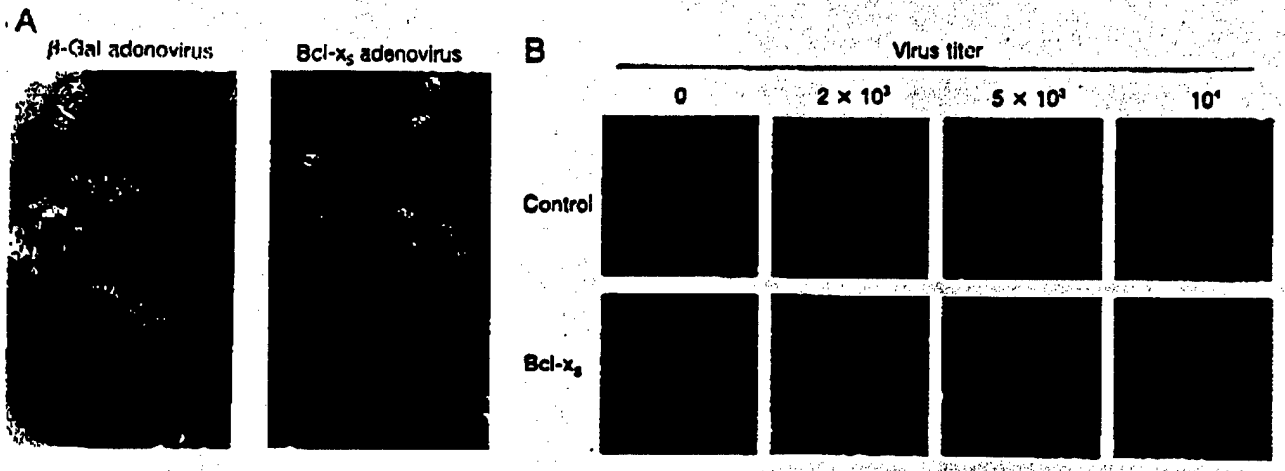

FIG. 2. (*A*) Microphotograph of MCF-7 cells. MCF-7 cells (5×10^5) infected with the indicated virus photographed after 6 days of growth. β-Gal, β-galactosidase. Note that virtually all cells infected with *bcl-x$_S$* adenovirus have died. (×50.) (*B*) Photomicrograph of adenovirus infected primary breast cancer cells. Photomicrographs were taken of primary breast cancer cells infected 2 days previously with the indicated titer of β-galactosidase adenovirus (control) or the *bcl-x$_S$* adenovirus. Cells infected with even the lowest titer of *bcl-x$_S$* adenovirus show evidence of viral toxicity, whereas cells infected with even the highest titer of β-galactosidase virus remained viable. (×270.)

***bcl-x$_S$* Adenovirus Cytotoxicity Is Selective for Tumor Cells but Spares Human Hematopoietic Cells.** High-dose chemotherapy followed by infusion of autologous bone marrow to rescue the damaged hematopoietic system is felt to cure some children with neuroblastoma (26). Unfortunately, the bone marrow of such patients is often contaminated with neuroblastoma cells that contribute to relapse (27). To mimic the situation in which bone marrow cells collected for bone marrow transplantation are contaminated with cancer cells, 1×10^6 low-density human bone marrow mononuclear cells were mixed with 1.5×10^4 SHSY-5 human neuroblastoma cells. The lowest virus concentration (2×10^3 viruses per cell) completely inhibited the ability of SHSY-5 neuroblastoma cells to form colonies (data not shown). After exposure of the bone marrow cells to $2\text{-}10 \times 10^3$ viruses per cell, which totally inhibited proliferation of the neuroblastoma cells, human hematopoietic progenitor cells remained viable and formed colonies in methylcellulose (Fig. 3). There was a slight decrease in hematopoietic cell colonies after exposure to 1×10^4 viruses per cell. This result was not specific for the *bcl-x$_S$* adenovirus because it was observed with a control adenovirus (data not shown) and is probably secondary to nonspecific viral particle toxicity at very high doses (B.D., unpublished observation).

Table 1. Viability of different cancer cells after exposure to *bcl-x$_S$* adenovirus

Cells	Cytotoxicity: β-Galactosidase adenovirus	Cytotoxicity: *bcl-x$_S$* adenovirus
RKO (human colon carcinoma)	−	++++
Primary breast carcinoma cells*		
Patient 1		+++
Patient 2	−	+++
Patient 3	+	++++
Patient 4		+++
Patient 5	+	+++
Patient 6		++++
MDA435 (human breast carcinoma)	+++	++++
T47D (human breast carcinoma)	+++	++++
MCF-7 (human breast carcinoma)	++	+++
HT29 (human colon carcinoma)	++	++++
SHSY-5 (human neuroblastoma)	+	++++
SK-N-SH (human neuroblastoma)	+	++++
IMR-32 (human neuroblastoma)	+++	+++
K-562 (human leukemia)	−	−

The indicated cell lines were infected with *bcl-x$_S$* adenovirus by using a virus titer that resulted in expression of β-galactosidase in >95% of cells infected with the same titer of β-galactosidase virus. In cell lines, each experiment was done in triplicate, and viability was determined 6 days after infection. Degree of cytoxicity was as follows: − (<5%), + (6–25%), ++ (26–50%), +++ (51–90%), and ++++ (91–100%). *Breast cancer cells isolated from either pleural or ascites fluid were collected by the University of Michigan tissue procurement laboratory and stored in liquid nitrogen. These cells were placed in tissue culture medium and exposed to the *bcl-x$_S$* adenovirus. The primary breast cancer cells were infected with identical titers of either β-galactosidase or *bcl-x$_S$* adenovirus that resulted in expression of β-galactosidase in most cells. The primary breast cancer cells infected with β-galactosidase virus remained viable.

Hematopoietic Cells Exposed to the *bcl-x$_S$* Adenovirus Retained the Ability to Reconstitute Bone Marrow. For the *bcl-x$_S$* adenovirus to be clinically effective in tumor cell purging, human hematopoietic stem cells capable of repopulating the patient must be spared. Recently, transplantation assays for primitive human SCID-repopulating cells (21) have been developed by engrafting human bone marrow or cord blood in irradiated immune-deficient SCID or Nod/SCID mice (22,

Table 2. Tumor formation in nude mice

Adenovirus	Mice injected, no.	Tumors, no.
Mock	10	7
β-Galactosidase	5	2
bcl-x$_S$	15	0

RKO colon cancer cells were infected with 10^4 of the indicated adenovirus per cell. Approximately 16 hr later, 5×10^6 cells were collected and injected into the flanks of nude mice. Control cells (either mock-infected cells or β-galactosidase adenovirus-infected cells) were injected into one flank, and *bcl-x$_S$* adenovirus-infected cells were injected into the opposite flank of 15 mice. Mice were examined 4 weeks later for tumors. Statistical analysis using the Wilcoxon signed-rank analysis shows a significant difference in the number of tumors that the control cells vs. the *bcl-x$_S$* adenovirus-infected cells formed ($P = 0.018$).

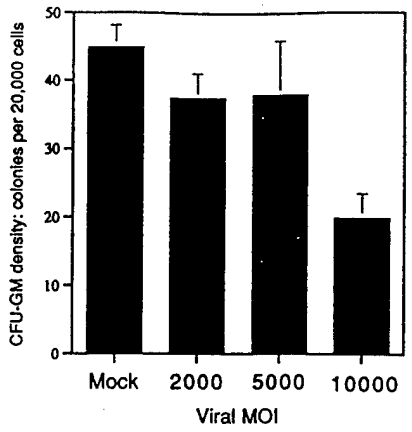

FIG. 3. Mononuclear cells from normal human bone marrow were isolated as described (20). Duplicate samples of hematopoietic cells were infected with the indicated number of *bcl-x$_S$* adenoviruses per cell, and then progenitor assays were done in triplicate. Note that only at the highest virus concentration is there any decline in colony numbers. Data from one experiment are shown. A second experiment using a different donor yielded essentially identical results. MOI, multiplicity of infection.

28). To ensure that SCID-repopulating cells remained functional after exposure to the *bcl-x$_S$* adenovirus, treated human bone marrow cells were transplanted into Nod/SCID mice. Human bone marrow mononuclear cells were exposed to up to 1×10^5 *bcl-x$_S$* adenovirus vector per cell and cultured *in vitro*. The hematopoietic cells (1×10^7) were then infused into the tail vein of irradiated mice according to standard protocols (21, 22). One month after transplantation, DNA analysis with a human-specific α-satellite probe indicated that significant levels of human cells had repopulated the mouse bone marrow (Table 3). In addition, the bone marrow contained multiple lineages of human myeloid and erythroid progenitors, even in mice transplanted with cells exposed to the highest virus titer (Table 3). Quantitatively and qualitatively, these mice were indistinguishable from several hundred mice that we have transplanted with normal human cells, indicating that the SCID-repopulating cells were unaffected by exposure to the *bcl-x$_S$* adenovirus. These data suggest the feasibility of using the *bcl-x$_S$* adenovirus vector to eliminate cancer cells from the bone marrow while sparing normal stem cells.

DISCUSSION

It has been postulated that Bcl-2 may contribute to the malignant phenotype by blocking apoptotic pathways in cancer cells. In this present report, we used an adenovirus vector containing *bcl-x$_S$*, a functional inhibitor of Bcl-2, to induce PCD in human cancer cells derived from a variety of solid tumors. Furthermore, our evidence shows that the cytotoxicity induced by the *bcl-x$_S$* adenovirus vector is cell-type specific because normal human bone marrow hematopoietic progenitor cells are resistant to *bcl-x$_S$* adenovirus-induced apoptosis. Indeed, human hematopoietic cells exposed to these viruses retained the ability to reconstitute the bone marrow of irradiated SCID mice.

Initial descriptions of Bcl-x$_S$ suggested that expression of this protein inhibited the ability of Bcl-2 to protect cells from PCD induced by interleukin 3 withdrawal (15). It is notable that the *bcl-x$_S$* adenovirus is uniformly lethal to all solid tumor cells thus far tested. In contrast, the *bcl-x$_S$* adenovirus failed to induce cell death in hematopoietic precursors. The mechanism for this cell-type specificity is presently unknown. Expression of *bcl-x* is obligate for fetal liver hematopoiesis (29). However, it is not known whether expression of Bcl-x$_L$ is necessary for adult hematopoiesis or at what stage of differentiation expression of *bcl-x$_L$* is required for survival. The cell-type selectivity of the *bcl-x$_S$* adenovirus may, at least partly, be due to the fact that these recombinant adenoviruses do not result in prolonged expression of transgenes in hematopoietic stem cells and that expression of Bcl-x$_L$ is not required for such cells to survive. It is also possible that the *bcl-x$_S$* adenovirus does not infect stem cells. Recent evidence shows that adenovirus vectors demonstrate tissue specificity. In lung tissue recombinant adenoviruses do not efficiently transduce columnar epithelial cells *in vivo* (30).

The observation that the *bcl-x$_S$* adenovirus is uniformly toxic to such a diverse number of cancer cells suggests that expression of a *bcl-2* family member may be obligate for cell survival in cancer cells of solid tissue origin. It has been thought that Bcl-2 and Bcl-x$_L$ proteins protect cells from apoptotic signals such as those induced by growth factor withdrawal or DNA damage (8, 9). Our results suggest that such signals might be constitutively present in certain cells. Cells stably transfected with a *bcl-x$_S$* plasmid and grown using selection medium in culture (15) uniformly express a small amount of Bcl-x$_S$ protein compared with cells infected with the *bcl-x$_S$* adenovirus (G.N., unpublished data). Thus, efficient induction of apoptosis by

Table 3. Human hematopoietic cell engraftment of SCID mice

	Human cells, %	Colonies					
			CFU-				
		BFU-E	G	M	GM	GEMM	Total
Mock infection	1–10	3	27	18	0	1	49
Mock infection	0	1	4	0	0	0	5
2K virus infection	10–50	22	43	81	4	1	151
2K virus infection	10–50	38	77	157	4	2	278
5K virus infection	1–10	2	9	13	0	0	24
5K virus infection	N/A	—	—	—	—	—	—
10K virus infection	1–10	2	15	24	1	1	43
10K virus infection	1–10	2	17	16	2	0	37

Low-density mononuclear cells from human bone marrow were collected as described (20) and infected with the indicated number of the *bcl-x$_S$* adenoviruses per cell. The next day, irradiated SCID mice were injected with $\approx 1 \times 10^7$ cells essentially as described (21, 22). After 1 mo, bone marrow cells were harvested. Southern blots were done to determine the percentage of human cells in the bone marrow (22). Low-density mononuclear cells were cultured in duplicate in methylcellulose with human hematopoietic growth factors, and erythroid (BFU-E), granulocyte (CFU-G), macrophage (CFU-M), granulocyte/macrophage (CFU-GM), and mixed granulocyte/erythroid/monocyte (CFU-GEMM) colonies were counted 2 weeks later. One of the mice injected with cells that were exposed to 5×10^5 (5K) viruses per cell died before analysis. Note that mouse marrow was engrafted with human hematopoietic cells exposed to the highest titer of virus.

the *bcl-x$_S$* adenovirus may relate to its ability to transduce high levels of Bcl-x$_S$ protein in infected cells. An alternative explanation is that cancer cells infected with the *bcl-x$_S$* adenovirus express an effector of apoptosis, either an endogenous effector or a virally encoded effector (or both).

Cancer cell contamination of bone marrow used to rescue patients from high-dose chemotherapy is a significant problem in the treatment of neuroblastoma (31) and breast cancer (32). Elegant retrovirus-tagging experiments have shown that reinfusion of malignant cells contributes to the relapse of neuroblastoma (27). In all relapsed patients, biopsies of such tumors showed that virally marked cells were invariably present (27). We have shown that after infection of contaminated bone marrow cells with the *bcl-x$_S$* adenovirus, the cells can be incubated *in vitro* for a short period to allow the carcinoma cells to die and then be infused into a mouse and reconstitute hematopoiesis. By this method, the *bcl-x$_S$* adenovirus can be used as a "molecular scalpel," either by itself or in conjunction with other purging techniques (33), to selectively eliminate contaminating tumor cells from bone marrow samples. Together, these observations suggest that strategies such as the *bcl-x$_S$* adenovirus designed to disrupt the *bcl-2* family pathway may provide alternative therapeutic approaches to cancer treatment.

We thank Dr. Mark Roth for many helpful discussions and dedicate this manuscript to his memory. We thank Dr. S. Ethier for supplying primary breast carcinoma cells, Dr. C. Croce for providing the MCF-7 mutant p53 cell line, Dr. M. Kastan for providing the RKO cell line, and Dr. V. Castle for supplying the SHSY-5 cell line. This work was supported by Grants CA46657, CA64556-01, and the University of Michigan Cancer Center Grant CA46592 from the National Cancer Institute; by Department of Defense Grants DAMD17-95-1-5021 and 17-94-5-4382; by a grant from the Charlotte Geyer Foundation; and by a grant from the American Chemical Society. J.E.D. is supported by grants from the National Cancer Institute of Canada (NCIC), the Medical Research Council of Canada, and the Genetic Diseases Network of the Centers of Excellence. J.E.D. is a Research Scientist of the NCIC. G.N. is supported, in part, by Research Career Development Award CA64421-01 from the National Institutes of Health.

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Advances in Brief

bcl-x$_s$ Gene Therapy Induces Apoptosis of Human Mammary Tumors in Nude Mice[1]

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Abstract

Bcl-x$_s$ is a dominant negative repressor of Bcl-2 and Bcl-x$_L$, both of which inhibit apoptosis. We used a replication-deficient adenoviral vector to transiently overexpress Bcl-x$_s$ in MCF-7 human breast cancer cells, which overexpress Bcl-x$_L$. Infection with this vector induced apoptosis *in vitro*. We then determined the effects of intratumoral injection of *bcl-x$_s$* adenovirus on solid MCF-7 tumors in nude mice. Tumors injected four times with the *bcl-x$_s$* adenovirus showed a 50% reduction in size. Using terminal transferase-mediated dUTP-digoxigenin nick end labeling, we observed apoptotic cells at sites of *bcl-x$_s$* adenoviral injection. These experiments demonstrate the feasibility of using *bcl-x$_s$* gene therapy to induce apoptosis in human breast tumors.

Introduction

Overexpression of genes that inhibit programmed cell death (apoptosis) may play a role in the etiology of a variety of cancers (1), including breast cancer. Genes in these pathways, therefore, may represent novel targets for cancer therapy. The proto-oncogene *bcl-2* is overexpressed in up to 70% of breast cancers (2) and may be an important negative regulator of apoptosis in these cancers. The Bcl-2 protein was first described as being overexpressed in follicular lymphomas, in which it participates in tumor formation by blocking cell death (3, 4). The ability of Bcl-2 overexpression to inhibit apoptosis has been verified *in vivo* using transgenic mice (5). A genetic homologue of *bcl-2* named *bcl-x* has been cloned. Due to alternate mRNA splicing, the *bcl-x* gene is transcribed into long (*bcl-x$_L$*) and short (*bcl-x$_s$*) forms (6). The Bcl-x$_L$ protein, like Bcl-2, functions as an inhibitor of apoptosis (6–8). Overexpression of Bcl-x$_L$ has been shown to protect human neuroblastoma cells from apoptosis induced by the chemotherapeutic agents 4-hydroperoxycyclophosphamide and cisplatin (7). In contrast, the Bcl-x$_s$ protein functions as a dominant negative repressor of Bcl-2 and Bcl-x$_L$ (6, 9). We have previously reported that 4-fold overexpression of Bcl-x$_s$ in MCF-7 human breast cancer cells, which express Bcl-2 and overexpress Bcl-x$_L$, sensitized these cells to apoptosis induced by the chemotherapeutic agents etoposide and taxol (10). The inability to produce stably transfected clones that overexpress Bcl-x$_s$ by more than 4-fold suggests that higher levels of Bcl-x$_s$ may be lethal to cells. Therefore, we hypothesized that transient overexpression of large amounts of Bcl-x$_s$ would induce apoptosis in the absence of other insults. To test this, we used adenovirus-mediated transfer of a *bcl-x$_s$* minigene to transiently overexpress high levels of Bcl-x$_s$ in MCF-7 cells. We report that this transduction caused apoptosis *in vitro*. In other systems, such as prostate and head and neck cancer, adenoviral-mediated transfer of the tumor suppressor genes *p53* and *p21* have been shown to inhibit solid tumor growth *in vivo* (11, 12). Based on our *in vitro* data, we have extended our studies to examine the effect of adenovirus-transduced *bcl-x$_s$* on MCF-7 tumor growth *in vivo*. We report that such *bcl-x$_s$* gene therapy retards the growth of solid MCF-7 tumors in nude mice by the induction of apoptosis *in vivo*.

Materials and Methods

Construction of *bcl-x$_s$* and *lacZ* Adenoviruses. The *bcl-x$_s$* minigene was excised from the plasmid pBS*bcl-x$_s$* (provided by G. Nuñez; Ref. 6). The bacterial *lacZ* gene (with nuclear localization sequence) or *bcl-x$_s$* minigene was inserted into the *Bam*HI site of the pAd5RSV plasmid. This plasmid was derived by inserting the RSV[3] promoter into the *Bgl*2 site of the pAdBgl2 vector.[4] The *bcl-x$_s$* and *lacZ* recombinant adenoviruses were isolated by *in vivo* homologous recombination between the pAd5RSV*bcl-x$_s$* or pAd5RSV*lacZ* plasmid and replication-deficient sub360 adenovirus, which has a deletion of the *E1A* and *E1B* genes, as described (9). Large preparations of the adenovirus were made by infecting 293 kidney cells and purifying crude virus by cesium chloride centrifugation. The viral titer was quantified by spectrophotometry.

Growth Studies of MCF-7 Cells. MCF-7 human breast cancer cells were cultured in MEM plus L-glutamine (Celox, Hopkins, MN) supplemented with 10% fetal bovine serum, 10 μg/ml insulin, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1% nonessential amino acid solution (Sigma Chemical Co., St. Louis, MO). For growth studies, MCF-7 cells were first plated at 10^4 cells/well for 24 h, then treated with serum-free MEM containing the *bcl-x$_s$* or *lacZ* adenovirus at 2–5 × 10^3 pfu/cell for 3 h. This medium was replaced with MEM plus insulin plus 4% fetal bovine serum overnight, then full culture medium. Cell growth was assessed by cell counts using a Coulter counter (Coulter Electronics, Inc., Hialeah, FL) and by hemocytometry.

Western Blot Analysis. Protein samples were resolved by denaturing SDS-PAGE and Western blotted by standard methods. Bcl-x$_L$ and Bcl-x$_s$ were detected using a rabbit polyclonal IgG to human Bcl-x (Santa Cruz Biotechnology, Santa Cruz, CA). A goat antirabbit antibody conjugated to horseradish peroxidase and enhanced chemiluminescence (Amersham, Arlington Heights, IL) were used to visualize protein bands.

Quantitation of Apoptosis by ELISA. To quantitate the relative number of apoptotic cells *in vitro*, we used an ELISA (Boehringer Mannheim, Indianapolis, IN), which measures cytoplasmic DNA-histone complexes generated during apoptotic DNA fragmentation, as described previously (10).

Growth of MCF-7 Solid Tumors in Nude Mice. Six-week-old, female, athymic nude mice (CD-1, *nu/nu*; Charles River Breeding Laboratories, Wilmington, MA) were implanted with 0.72 mg 17β-estradiol pellets (6-week time release; Innovative Research of America, Sarasota, FL). Two days later, 6×10^6 MCF-7 cells were injected s.c. in 0.1 ml 50% matrigel (Collaborative Biomedical Products, Bedford, MA) plus 50% unsupplemented MEM, as described (13). This method has yielded a 95% success rate for the formation of MCF-7 tumors *in vivo*.

Adenoviral-mediated Bcl-x$_s$ Overexpression *in Vivo*. One of two tumors per mouse was treated by direct intratumoral injection with the *bcl-x$_s$* adenovirus, and the other was injected with the *lacZ* adenovirus, using a 0.1-ml syringe with a 26-gauge, 0.5-inch needle. A single needle puncture was made into the skin covering the tumor; then, the needle tip was moved to five

Received 1/18/96; accepted 3/15/96.

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This research was supported by NIH Grants CA-61777 and CA-64556 and American Cancer Society Grant BE-161C.

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[1] The abbreviations used are: RSV, Rous sarcoma virus; pfu, plaque forming unit; gal, galactosidase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end labeling.

[4] B. Davidson, personal communication.

different sites within the tumor, and 10 μl adenovirus preparation were injected at each site. Tumor volume was measured every 3–4 days. Linear calipers were used to measure the longest axis (a) and the width perpendicular to this axis (b). Tumor volume was calculated as: volume = $a \times b^2 \times 0.4$.

X-gal Staining and Detection of Apoptosis by TUNEL. Mice were sacrificed, and tumors were frozen in OCT embedding medium (Miles, Inc., Elkhart, IN) by standard methods. β-Gal activity was detected by X-gal staining on 20-μm cryosections as described (14). Internucleosomal DNA cleavage characteristic of apoptotic cells was detected *in situ* on 8-μm cryosections by TUNEL (ApopTag kit; Oncor, Gaithersburg, MD) as described (15).

Statistics. For ELISA data and *in vitro* cell counts, statistical significance was calculated by Student's paired t test. For *in vivo* tumor growth experiments, overall statistical significance between control and treated groups was calculated by repeated-measures analysis using Statistical Analysis System software (SAS Institute, Inc., Cary, NC). P values are given in each figure legend.

Results

Overexpression of Bcl-x_s by an Adenoviral Vector Induces Apoptosis of MCF-7 Human Breast Cancer Cells *in Vitro*. Expression of Bcl-x family members by MCF-7 cells was assayed by Western blotting using a rabbit polyclonal antibody that recognizes both Bcl-x_L (M_r 25,630) and Bcl-x_s (M_r 18,700). As shown in Fig. 1*A*, *Lanes 3* and *4*, control MCF-7 cells exclusively express Bcl-x_L and do not express Bcl-x_s. The effects of transient overexpression of Bcl-x_s on MCF-7 cells *in vitro* were tested by infecting these cells with an adenovirus containing a *bcl-x_s* minigene (pAdRSV-*bcl-x_s*). Another adenovirus carrying the bacterial gene for β-gal (pAdRSV-*lacZ*) was used as a control. As shown in Fig. 1*A*, *Lanes 1* and *2*, MCF-7 cells infected 3 days previously with 2×10^3 pfu/cell *bcl-x_s* adenovirus express Bcl-x_s in addition to Bcl-x_L.

We next determined the effect of Bcl-x_s overexpression on cell viability in culture. Beginning 2 days after infection, large numbers of dead, floating cells were observed in MCF-7 cultures infected with the *bcl-x_s* adenovirus. Fig. 1*B* shows that 5 days after infection with 10^4 pfu/cell, the *bcl-x_s* adenovirus caused a 45% greater reduction in the number of viable cells, as determined by trypan blue exclusion, than the *lacZ* adenovirus ($P = 0.010$; $n = 3$). To determine whether these differences in viable cell numbers were caused by apoptosis induced by the *bcl-x_s* adenovirus, we used an ELISA. This assay quantitates cytoplasmic, histone-bound DNA derived from internucleosomal DNA fragmentation, which occurs in the nuclei of apoptotic cells. Table 1 shows that cells infected with 2×10^3 pfu/cell *bcl-x_s* adenovirus had 6-fold greater cytoplasmic DNA-histone than uninfected controls and 4.6-fold greater DNA-histone than *lacZ* controls ($P \leq 0.01$; $n = 3$). These data suggest that the *bcl-x_s* adenovirus, specifically, induces apoptosis in MCF-7 cells, accounting for the observed decrease in viability.

We then tested whether the MCF-7 cells which remained viable by trypan blue exclusion after *bcl-x_s* infection were clonogenic. To accomplish this, we tested the ability of *bcl-x_s*- or *lacZ*-infected MCF-7 cells to grow as solid, s.c. tumors in estrogen-supplemented nude mice. Three $\times 10^7$ pfu *bcl-x_s* or *lacZ* adenovirus were added to 3×10^6 MCF-7 cells immediately prior to s.c. injection into the flank of a mouse. Fig. 1*C* shows that although the *bcl-x_s*-infected cells formed a small nodule, the nodule did not grow ($P = 0.0085$; $n = 2$). The *lacZ*-infected cells formed larger nodules, which, after a growth delay, began to grow at a rate similar to that in uninfected controls. After 25 days, *bcl-x_s*-infected tumors were 91% smaller than *lacZ*-infected controls. These results indicate that the MCF-7 cells which excluded trypan blue following *bcl-x_s* infection were incapable of growing as a tumor *in vivo*, suggesting that these cells were no longer clonogenic. When titers higher than 3×10^7 pfu were tested, the adenovirus vector alone was toxic to the cells, and tumors did not form.

***In Vivo bcl-x_s* Adenoviral Treatment Retards the Growth of MCF-7 Tumors in Nude Mice.** To determine whether the *bcl-x_s* adenoviral vector would be useful for *in vivo* gene therapy, we extended our study to see whether *in vivo bcl-x_s* adenoviral treatment would retard the growth of established tumors. Three-day-old MCF-7 tumors in estrogen-supplemented nude mice were treated by single injections of 7×10^7 pfu *bcl-x_s* or *lacZ* adenovirus. Fig. 2*A* shows that tumors treated with the *bcl-x_s* adenovirus grew at a decreased rate for 8 days after treatment, then began to grow at a rate similar to *lacZ*-infected tumors ($P = 0.031$; $n = 3$). Tumors treated with the *lacZ* adenovirus grew at a rate similar to that of untreated controls. The greatest *bcl-x_s*-specific tumor growth inhibition was observed 8

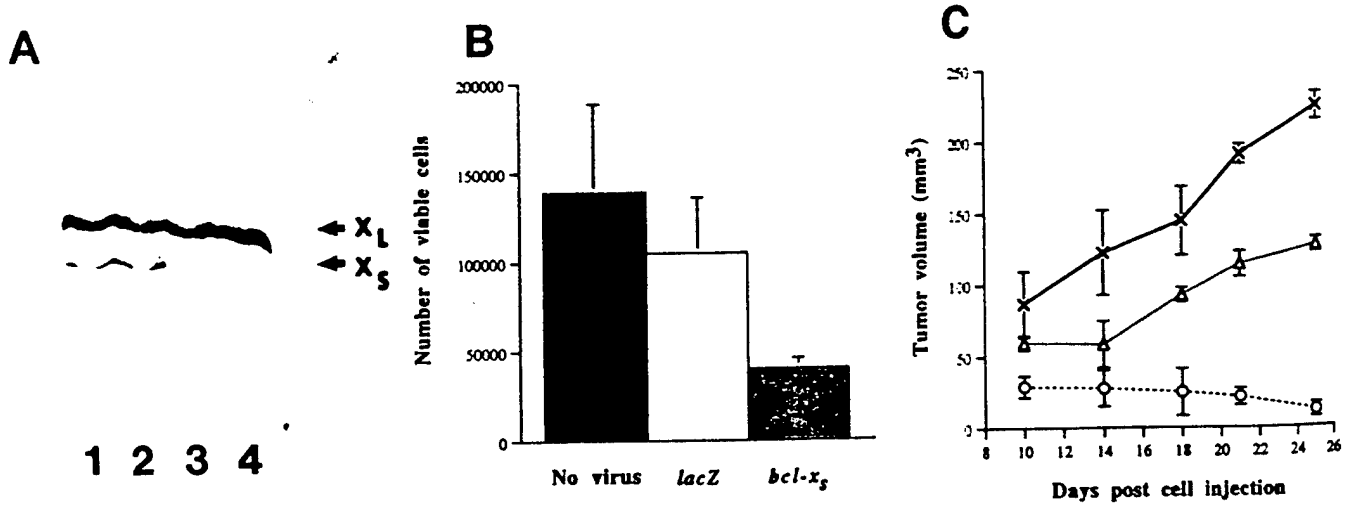

Fig. 1. Effects of Bcl-x_s expression on cell viability. *A*, Western blot of Bcl-x expression. MCF-7 cells were infected as described in "Materials and Methods" with 2×10^3 pfu/cell *bcl-x_s* or *lacZ* adenovirus, and total protein lysates (400 μg/lane) were analyzed by Western blotting using a rabbit polyclonal antibody to Bcl-x. *Lanes 1* and *2*, *bcl-x_s*-infected cells; *lanes 3* and *4*, *lacZ*-infected cells. *Arrows*: x_L, Bcl-x_L (M_r 25,630); x_s, Bcl-x_s (M_r 18,700). *B*, effect of *bcl-x_s* adenoviral infection on MCF-7 viability. MCF-7 cells were infected *in vitro* with 10^4 pfu/cell *bcl-x_s* or *lacZ* adenovirus. The number of live cells, as determined by trypan blue exclusion, was counted 5 days later ($P = 0.010$; *bars*, SE; $n = 3$). *C*, effect of *bcl-x_s* adenoviral infection on clonogenicity of MCF-7 cells. MCF-7 cells (3×10^6) were treated with 10 pfu/cell *bcl-x_s* or *lacZ* adenovirus *in vitro* and injected s.c. into opposite flanks of an estrogen-supplemented nude mouse. Tumor volume was calculated as described in "Materials and Methods" ($P = 0.0085$; *bars*, SD; $n = 2$). ×, no virus; △, *lacZ*; ○, *bcl-x_s*.

Table 1 *Quantitation of apoptosis by ELISA*

MCF-7 cells were infected with the *bcl-x*$_s$ or *lacZ* adenovirus (2×10^3 pfu/cell), and after 6 days cytoplasmic extracts from equal total numbers of cells (live and dead) were assayed for DNA-histone as described (10). Values are normalized to 1.0 for uninfected MCF-7 cells ($P \leq 0.01$; $n = 3$).

Sample	Mean ± SE units of cytoplasmic DNA-histone
Uninfected MCF-7	1.00
bcl-x$_s$-infected MCF-7	6.07 ± 1.94
lacZ-infected MCF-7	1.32 ± 0.25

days after treatment, when *bcl-x*$_s$-infected tumors were 38% smaller than *lacZ*-infected tumors. This experiment showed that *in vivo bcl-x*$_s$ adenoviral infection retarded tumor growth, but that single treatment was not sufficient for long-term growth inhibition.

We hypothesized that, relative to *in vitro* infection, *in vivo* infection had less effect on tumor growth because only a fraction of the cells was infected; this hypothesis was subsequently confirmed (see Fig. 3*A*). Therefore, we tested whether multiple *in vivo* infections with the *bcl-x*$_s$ adenovirus would cause greater or sustained retardation of tumor growth. Three-day-old MCF-7 tumors were treated with 7×10^7 pfu *bcl-x*$_s$ or *lacZ* adenovirus every 4 days for five treatments. Fig. 2*B* shows that throughout the duration of treatment, the *bcl-x*$_s$-infected tumors grew at a slower rate than the *lacZ*-infected tumors ($P = 0.0032$; $n = 4$). The greatest *bcl-x*$_s$-specific retardation of growth was observed following four treatments (13 days after the first treatment), when *bcl-x*$_s$-infected tumors were 52% smaller than *lacZ*-infected tumors. Thus, multiple treatments with the *bcl-x*$_s$ adenovirus caused greater and sustained tumor growth inhibition relative to single treatment.

Inhibition of MCF-7 Tumor Growth *in Vivo* by *bcl-x*$_s$ Adenoviral Infection Is Due to Induction of Apoptosis. To determine the efficacy of our direct intratumoral approach to adenoviral-mediated gene therapy, we estimated the number of cells in an infected tumor that expressed the transduced gene, using *lacZ* as a marker. Three-day-old MCF-7 tumors were treated *in vivo* with 7×10^7 pfu *lacZ* adenovirus, and expression of the viral-encoded gene was detected 4 days later by X-gal staining. Overall, about 15% of the tumor cells expressed the transduced *lacZ* gene ($n = 2$). As seen in Fig. 3*A*, we found a higher concentration of transduced cells along linear tracks, presumably at sites of needle injection. Thus, gene transfer was successful to a portion of the tumor cells.

To determine whether cells infected *in vivo* with the *bcl-x*$_s$ or *lacZ* adenovirus were apoptotic, X-gal staining was used to locate infected cells; then, apoptotic cells were detected on serial sections by TUNEL. Fig. 3*B* shows a TUNEL assay of a serial section of the *lacZ*-infected tumor seen in Fig. 3*A*. The absence of apoptotic cells in the infected region of the tumor shows that the *lacZ* adenovirus did not induce apoptosis ($n = 2$). To determine whether the *bcl-x*$_s$ adenovirus induced apoptosis in transduced cells, we used a combined approach, in which 90% *bcl-x*$_s$ adenovirus was mixed with 10% *lacZ* adenovirus as a tracer. This approach allowed us to determine areas of successful gene transfer using *lacZ* as a marker while analyzing the effects of *bcl-x*$_s$ on apoptosis. Fig. 3, *C* and *D*, shows X-gal staining and TUNEL of a tumor treated *in vivo* with 90% *bcl-x*$_s$ plus 10% *lacZ* adenovirus. In contrast to treatment with *lacZ* alone, the addition of the *bcl-x*$_s$ adenovirus caused apoptosis in the infected area of the tumor ($n = 2$). This is seen more clearly in Fig. 3*E*, which shows apoptotic nuclei visible at a higher magnification. These experiments demonstrate that *in vivo bcl-x*$_s$ adenoviral treatment induced cell death by apoptosis, consistent with the inhibition of the overall tumor growth rate caused by this treatment.

Discussion

Normal mammary epithelial cells are capable of undergoing apoptosis during involution following lactation (16). Inhibitors of this ability to undergo apoptosis, such as Bcl-2 and Bcl-x_L, may play an important role in the development of breast cancer. In this communication, we report that MCF-7 human breast cancer cells overexpress Bcl-x_L but do not express Bcl-x_s. Bcl-x_s is a dominant negative repressor of Bcl-2 and Bcl-x_L and is, therefore, a putative inducer of apoptosis in cancer cells which are resistant to apoptosis due to overexpression of Bcl-2 and/or Bcl-x_L (6, 9). We report that adenovirus-mediated overexpression of Bcl-x_s induces apoptosis of MCF-7 human breast cancer cells *in vitro*. In addition, we report that adenovirus-mediated *bcl-x*$_s$ gene therapy can retard MCF-7 tumor growth in a nude mouse model and present evidence that this growth inhibition is due to induction of apoptosis. The *bcl-x*$_s$ adenovirus, which is replication deficient, caused no obvious systemic or local toxicity, such as skin necrosis, when delivered locally.

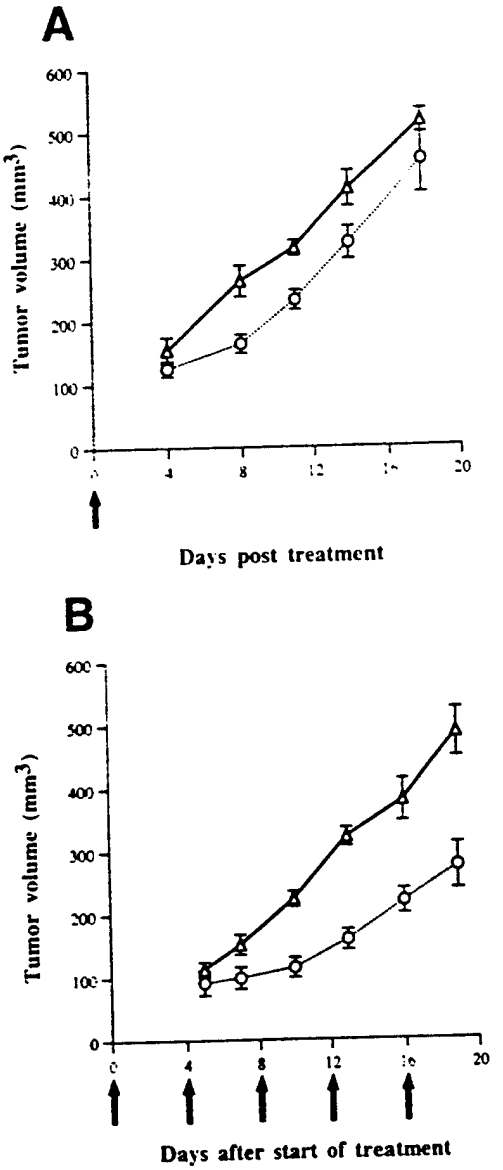

Fig. 2. *bcl-x*$_s$ adenoviral effects on tumor growth *in vivo*. *A*, tumor growth following single injection. Three days after s.c. injection of 6×10^6 MCF-7 cells, tumors were injected with 7×10^7 pfu *bcl-x*$_s$ or *lacZ* adenovirus ($P = 0.031$; *bars*, SE; $n = 3$). *B*, tumor growth following multiple injections. Tumors were treated five times, once every 4 days ($P = 0.0032$; *bars*, SE; $n = 4$). *Arrows*, treatment. ↑, *LacZ*; ↑, *bcl-x*$_s$.

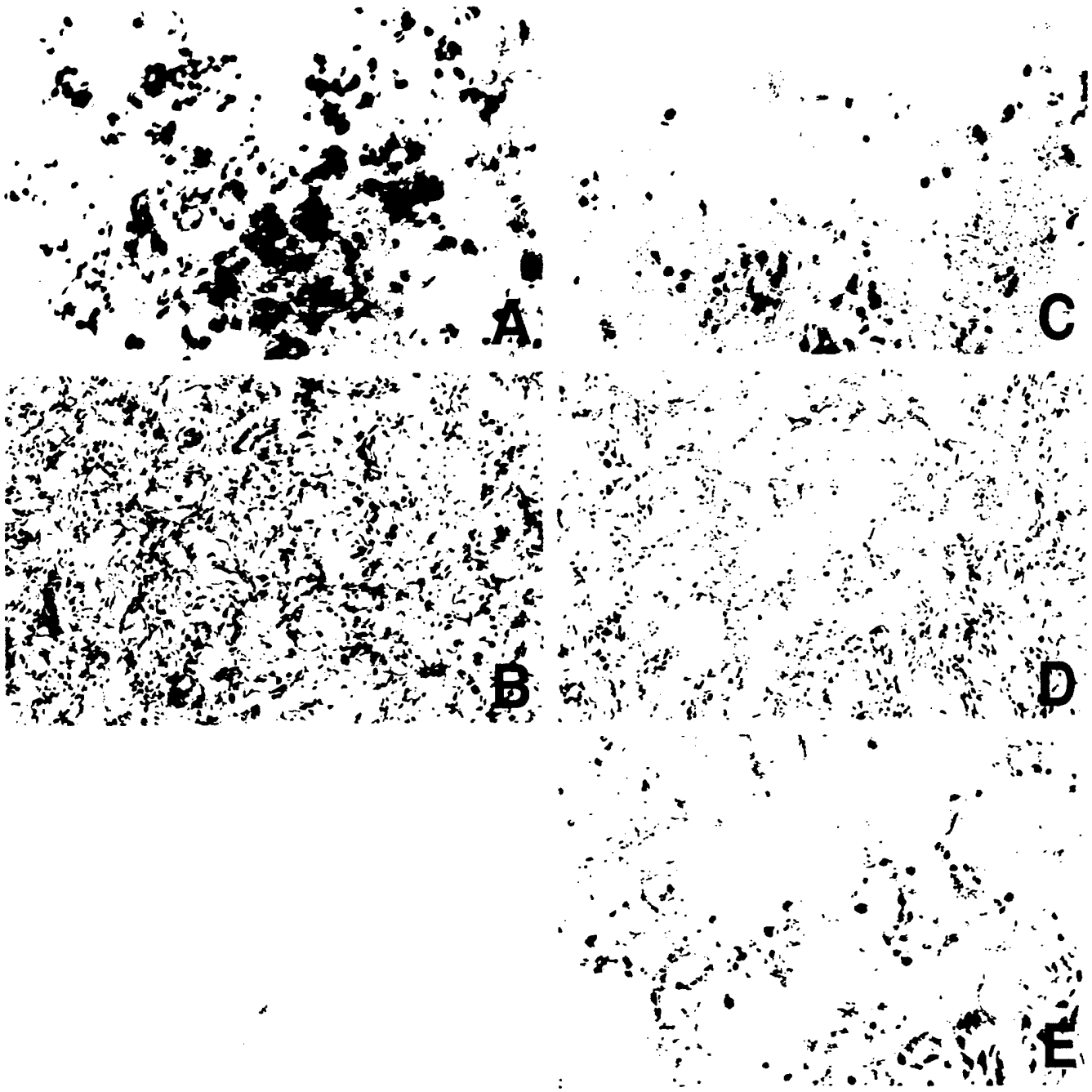

Fig. 3. Detection of gene expression and apoptosis *in situ*. Three-day-old MCF-7 tumors were treated with *lacZ* with or without *bcl-x*, adenovirus (7×10^7 pfu total) as indicated ($n = 2$). Four days later, mice were sacrificed, and tumors were assayed for *lacZ* gene transfer by X-gal staining (blue) and apoptosis by TUNEL (apoptotic cells appear brown). *A*, 100% *lacZ*, X-gal staining. *B*, 100% *lacZ*, TUNEL. *C*, 90% *bcl-x*, plus 10% *lacZ*, X-gal staining. *D*, 90% *bcl-x*, plus 10% *lacZ*, TUNEL. *E*, higher magnification of *D*.

In vivo bcl-x, adenoviral treatment reduced the size of the solid MCF-7 tumors by a greater amount than would be predicted from the percentage of cells that were infected as determined by *lacZ* expression. Single *in vivo* adenoviral treatment resulted in *bcl-x*,-infected tumors that were 38% smaller than *lacZ*-infected controls. However, X-gal staining showed that a single adenoviral treatment infected only about 15% of the cells in each tumor. This raises the possibility that *bcl-x*, adenoviral infection kills additional cells via a bystander effect, in which uninfected cells surrounding an infected cell also are killed. The bystander effect has been shown to induce apoptosis of unmodified tumor cells adjacent to cells transduced with the herpes simplex virus thymidine kinase gene and treated with ganciclovir (17). Such bystander killing was associated with the transfer of apoptotic vesicles from transduced, dying cells to adjacent untransduced cells (17). We are currently investigating whether Bcl-x_s-induced apoptosis may cause a similar bystander effect.

The *in vitro* ELISA data and *in situ* TUNEL assays presented here suggest that a significant fraction of the cell killing caused by the *bcl-x*, adenovirus is due to apoptosis. It is not yet clear whether this apoptosis is solely the result of Bcl-x_s overexpression, or whether the adenoviral vector presents an insult to the cell which contributes to the induction of apoptosis. However, the *bcl-x*, adenovirus caused significantly greater apoptosis than the same titer of the *lacZ* adenovirus, providing evidence that Bcl-x_s overexpression itself contributes to apoptosis and showing the importance of blocking inhibitors of cell death, such as Bcl-2 and Bcl-x_L. Furthermore, this shows that adeno-

viral-mediated gene transfer of *bcl-x*$_s$, which causes transient overexpression, is sufficient to induce apoptosis.

A possibility for improving the efficacy of *bcl-x*$_s$ gene therapy may come from a combination of this approach with chemotherapy. We have previously reported that stable transfection of *bcl-x*$_s$ sensitized MCF-7 cells to apoptosis induced by the chemotherapeutic agents etoposide and taxol *in vitro* (10). By blocking inhibitors of apoptosis, *bcl-x*$_s$ gene therapy may lower the apoptotic threshold of cancer cells to other agents that cause cell death.

Bcl-2 and Bcl-x_L, the functional targets of Bcl-x_s, seem to inhibit apoptosis through a common pathway (18). Bcl-2 has been shown to be overexpressed in up to 70% of breast cancers (2) and, therefore, may play an important role in the development of these cancers. Although such data have not yet been reported for Bcl-x_L, we have found Bcl-x_L to be overexpressed in a significant percentage of primary cell lines and tissues derived from breast cancers (data not shown). These data suggest that Bcl-2 and Bcl-x_L may be important targets for novel breast cancer treatments, such as *bcl-x*$_s$ adenoviral gene therapy.

Acknowledgments

We thank Gabriel Nuñez (University of Michigan Medical School, Ann Arbor, MI) for supplying the pBS*bcl-x*$_s$ plasmid and for many helpful discussions.

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A bcl-x_S adenovirus selectively induces apoptosis in transformed but not normal mammary cells

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Running Title: Therapeutic index of a bcl-x_S adenovirus

Key words: gene therapy, apoptosis, bcl-x_S , mammary cells

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** This work is supported by by NIH Grants CA 61777 and CA 66233

ABSTRACT

Oncogenes which drive the cell cycle such as c-myc, can sensitize cells to apoptosis upon growth factor withdrawal. This suggests the possibility that during carcinogenesis, the overexpression of genes such as bcl-2 or bcl-x_L are required to inhibit apoptosis induced by oncogene expression. We hypothesized that inhibition of Bcl-2/Bcl-xL by the pro-apoptotic Bcl-xS protein would result in selective induction of cell death in carcinoma cells compared to non-transformed cells. In order to test this hypothesis and to determine the therapeutic efficacy of a bcl-x_S adenovirus; we compared the effects of Bcl-x_S expression delivered by the bcl-x_S adenoviral vector, on non-transformed murine mammary cells (Comma-1D) and a c-myc transformed mouse mammary cell line (Myc-83). We found that whereas the non-transformed Comma-1D cells are resistant to the effects of the bcl-x_S vector, this vector efficiently induced apoptosis in the Myc-83 cells; consistent with the hypothesis that inhibition of Bcl-2 family of genes can result in selective killing of cancer cells compared to their non-transformed counterparts.

We extended these studies to a mouse breast cancer ascites model by demonstrating that the bcl-x_S adenoviral vector introduced intraperitoneally, reduces ascites formation and significantly prolongs survival with no detectable toxicity to normal mouse tissues. These studies demonstrate that the bcl-x_S adenovirus selectively kills transformed cells in vitro and in vivo, supporting the use of this approach for the gene therapy of breast cancer.

OVERVIEW SUMMARY

We have previously demonstrated the therapeutic potential of intra-tumoral adenoviral-bcl-x_S gene therapy for human breast cancers in a mouse model. We now report that this bcl-x_S adenovirus vector has differential effects on normal versus transformed mammary cells. This vector efficiently induced apoptosis in a c-myc transformed mouse mammary cell line (Myc-83), but not in normal Comma-1D mouse mammary cells. Both cell types were equally infectable with a lac-Z adenoviral vector, and expressed the Bcl-xS protein when infected with the bcl-x_S adenovirus. In a mouse model of breast cancer ascites, intraperitoneal administration of bcl-x_S adenovirus reduced ascites formation and prolonged survival with no apparent toxicity to normal cells. These studies show that Bcl-xS overexpression selectively kills transformed cells, suggesting a therapeutic index for this gene therapy approach.

INTRODUCTION

We and others have found that Bcl-2 and Bcl-xL which block apoptosis and promote cell survival, are frequently overexpressed in human breast cancers in vivo, as well as in primary cultures and established carcinoma cell lines (Silvestrini et al., 1994; Krajewski et al., 1994;). This suggests that antagonists of Bcl-2 and Bcl-xL may have utility as therapeutic agents in cancer. We have previously utilized Bcl-xS, a dominant negative inhibitor of Bcl-2 and Bcl-xL (Boise et al., 1993;), to target the apoptosis pathway in breast cancer cells. We showed that induction of low levels of Bcl-xS in breast cancer cells by stable transfection of plasmid encoded bcl-x_S, increased their sensitivity to apoptosis induced by chemotherapy (Sumantran et al., 1995;). Expression of higher levels of Bcl-xS via a bcl-x_S adenoviral vector directly induced apoptosis in breast carcinoma cells in vitro (Sumantran et al., 1995;), and caused tumor regression when administered intralesionally in vivo (Ealovega et al., 1996;).

A fundamental question concerning the utility of Bcl-2 inhibitors as therapeutic agents is the relative selectivity of apoptosis induction in carcinoma cells compared to untransformed cells from the same tissue. In other systems, oncogenes which drive the cell cycle such as c-myc, can sensitize cells to apoptosis upon growth factor withdrawal (Evan et al., 1992;). This suggests the possibility that during multistage carcinogenesis the overexpression of genes such as bcl-2 or bcl-x_L is obligate to inhibit apoptosis induced by oncogene overexpression. Thus, one might predict that inhibition of Bcl-2 would result in selective induction of cell death in carcinoma cells compared to non transformed cells. In order to test this hypothesis and to determine the therapeutic index of the bcl-x_S adenovirus vector as a gene therapy agent, we have compared the effects of Bcl-xS expression delivered by the bcl-x_S vector on untransformed murine mammary cells and a c-myc transformed mouse mammary cell line. This is clinically relevant because c-myc is overexpressed in up to 30% of human breast carcinomas (Berns et al., 1996;).

In this report we show that the c-myc transformed cells are extremely sensitive to apoptosis induced by the bcl-x_S adenovirus vector, whereas non-transformed murine mammary cells are resistant to programmed cell death induced by this vector. Furthermore, the bcl-x_S adenovirus introduced intraperitoneally, reduces ascites formation and prolongs survival in a mouse breast cancer ascites model, with no detectable toxicity to normal mouse tissue. These results are consistent with the hypothesis that transforming genes such as c-myc can sensitize cells to Bcl-xS induced apoptosis, resulting in a therapeutic index for bcl-2 antagonists. These results provide a rationale for utilizing the bcl-x_S adenovirus as an agent for gene therapy of breast cancer.

MATERIALS & METHODS

Cell types & Cell Culture

The c-myc transformed mouse mammary cell line was derived from breast adenocarcinomas developing in mice expressing a c-myc transgene. These cells are termed Myc-83 cells, and have been well characterized (Amundadottir et al., 1996;). The Comma-1D cells are normal mouse mammary cells which can differentiate and produce milk proteins in response to lactogenic hormones and extracellular matrix (Danielson et al., 1989;). This mouse mammary line is not tumorigenic in mouse models. Both cell lines were a kind gift from the laboratory of Dr. Robert Dickson (Lombardi Cancer Center, Georgetown University). These cell lines are cultured in Iscove's DMEM + 2.5% Fetal bovine serum (FBS) , 10 Ng/ml EGF, 5µg/ml Insulin, and antibiotics (Amundadottir et al., 1996;). The two cell types have similar plating efficiencies.

Adenoviral Vectors & Infection

The adenoviral-bcl-x_S vector was constructed as described (Clarke et al., 1995;). Bcl-xS expressing virus was purified from a single plaque that was bcl-x_S positive and E1A negative, as measured by the polymerase chain reaction (PCR). Viral batches derived from this batch were assayed for replication competent virus (RCA) by the quantitative plaque assay in A549 human lung carcinoma cells. The batches of bcl-x_S adenovirus used had RCA values of 1 in 10^5 to 1 in 3.0×10^6. The lac-Z adenovirus vector contains the E. coli gene lacZ (encoding β-galactosidase) under the control of an RSV promoter, also has an SV40 nuclear localization signal to enable easy detection of lac-Z adenoviral infected cells (Stratford-Perricaudet et al., 1992; Mastrangeli et al., 1993;). This vector had an RCA value of 1 in 2.5×10^4.

Cells were plated at 10^4/well in 12 well tissue culture plates and infected in serum free medium for 3-4 hours with either vector using a multiplicity of infection (MOI) of 250-2000, depending on the cell type. Infections were stopped by replacing virus with the appropriate growth medium. The percent adenoviral infection of cells was estimated by assaying lac-Z infected cells for β-galactosidase activity (Arakawa et al., 1991;) 2 days after infection.

Viability, Apoptosis, & Western blotting

Cell viability was determined by hemocytometry after trypan blue exclusion. A Cell Death ELISA assay was used to detect cytoplasmic DNA-histone complexes in apoptotic cells (Sumantran et al., 1995;). Protein lysates were prepared and resolved by SDS-PAGE (40 μg of protein/sample/lane) as described (Sumantran et al., 1995;). Western blotting was performed with a polyclonal rabbit antibody to human Bcl-x (Transduction Laboratories, Lexington, KY), which recognizes both Bcl-xL and Bcl-xS proteins. A goat-anti-rabbit antibody conjugated to peroxidase (Amresco, Solon, OH) and the ECL kit (Pierce, Rockford, IL) were used to detect protein bands.

In vivo experiments

MDA-MB-231 human breast carcinoma cells were cultured in RPMI + 10% FBS+ antibiotics. Six-eight week old female athymic nude mice (CD-1, nu/nu, purchased from Charles River Breeding Laboratories, Wilmington, MA) were injected intraperitoneally with MDA-MB-231 cells (10^7cells/mL in serum free medium) using a 23 gauge sterile needle. Ascites formation was monitored by weight gain and abdominal distension. The bcl-x_S versus lac-Z adenoviral gene therapy experiments were started when mice had gained 12-20% of their original body weight. Three viral injections were administered intraperitoneally once every 4 days with a 26 gauge needle using a viral titer of 3×10^9 MOI/injection. Mice were euthanized if high morbidity resulted from tumor progression, and this was recorded as the date of death.

In order to detect and quantitate viral gene transduction in vivo, ascites cells were aspirated with a 23 gauge needle 3 days after the mouse received an injection of either the bcl-x_S or lac-Z viral vector. The volumes aspirated ranged from 0.10-0.50 mls/mouse. Each aspirate on average contained 10^6 ascites cells/ml. The aspirates were diluted in media (RPMI + 10% FBS+ antibiotics), and plated for 24 hours. The cell layer were washed to remove red blood cells, and viable ascites cells from the aspirates of lac-Z injected mice were stained for β-galactosidase activity using the X-gal substrate (Arakawa et al., 1991;). The aspirates obtained from the bcl-x_S injected mice were treated similarly, and harvested for SDS-PAGE analysis. Western blotting was done to detect expression of the Bcl-xS protein.

Statistical Analysis

For in vitro data, statistical significance was determined by the Student's unpaired t-test. Standard errors and P values for a given data set are shown in the corresponding figure legends. For in vivo experiments, differences in survival of mice were analyzed by the log-rank test (Peto et al., 1972;). The survival distributions were estimated and displayed using the Kaplan-Meir Method (Kaplan et al., 1958;). Relative weight change (RWC) of mice is expressed as a percent change in weight of a mouse relative to its initial weight (weight on day of receiving tumor cells). RWC for the bcl-x_S treated, lac-Z treated, and control mice were plotted and modelled using a linear mixed effects statistical model (Littel et al., 1996;).

RESULTS

Adenoviral infectivity and Bcl-x_S protein expression in Comma-1D versus c-myc transformed mouse mammary cell lines

In order to compare the effects of Bcl-xS expression in transformed versus non-transformed mammary cells, we utilized two cell lines; the non-transformed murine mammary cell line, Comma-1D, and the c-myc transformed mouse mammary cell line, Myc-83. In the presence of the growth factors, insulin and EGF, these two cell lines grew at comparable rates (doubling time approximately 33 hours). Both cell types express endogenous Bcl-xL but not Bcl-xS (**Figure 2**).

In order to express transgenes in these cells we utilized adenoviral vectors. To compare the ability of adenoviral vectors to transduce these cells, we utilized a lac-Z adenovirus followed by X-gal staining to measure β-galactosidase expression. As shown in **Figure 1,** both the non-transformed Comma-1D mouse mammary cells (**Panel A**) and the transformed Myc-83 cells (**Panel B**) are efficiently transduced with the adenoviral vectors as shown by comparable levels of X-gal staining following lac-Z viral infection. Both cell types were 75%-80% infected with the lac-Z vector at an MOI of 1000. Furthermore, there were comparable levels of Bcl-xS protein expression following infection with the bcl-x_S adenovirus in the Comma-1D and the Myc-83 cells as determined by western blotting (**Figure 2:** lanes 2 and 5 respectively). These experiments demonstrate that adenoviral vectors are able to transduce both normal and transformed mammary cells in a similar manner.

Differential effects of Bcl-x_S expression on induction of apoptosis in normal versus c-myc transformed mammary cells

Despite comparable levels of Bcl-xS protein expression induced by bcl-x_S adenoviral infection in the Comma-1D and Myc-83 cells, there was a markedly different effect of the bcl-x_S vector on the viability and apoptosis in the two cell types. As shown in the photograph in **Figure 3**, the c-myc transformed murine mammary cells were efficiently killed by the bcl-x_S adenovirus four days after infection (Panel **f** vs **d**). In contrast, non-transformed Comma-1D mammary cells were highly resistant to bcl-x_S adenoviral killing (Panel **c** vs **a**). The control lac-Z adenovirus had little effect on the viability of either cell line (Panels **e** vs **d** for the Myc-83 cells; and Panels **b** vs **a** for the Comma-1D cells).

We examined the effects of various titers of bcl-x_S adenovirus, and times after infection, on the viability of both cell types. We found optimal cell killing of Myc-83 transformed cells at an MOI of 1,000. At

this titer killing was complete within two days. As can be seen in **Figure 4**, two days after infection, the bcl-x_S adenovirus induced greater than a 10-fold killing of the c-myc transformed cells compared to the control lac-Z adenovirus. In contrast, there was no specific killing of the non-transformed Comma-1D mammary cells by the bcl-x_S adenovirus vector under these conditions.

In order to demonstrate that the effects of bcl-x_S adenoviral infection on viability of the c-myc transformed cells resulted from induction of apoptosis, we utilized a cell death ELISA (Sumantran et al., 1995;). Both the non-transformed Comma-1D cells and the c-myc transformed cells exhibit similar, very low levels of apoptosis in the absence of adenoviral infection (**Table 1**). However, the bcl-x_S adenovirus induced a 12 fold greater increase in apoptosis in the Myc-83 cells compared to the level of apoptosis in bcl-x_S infected Comma-1D cells. Furthermore, when the ratio of apoptosis in bcl-x_S to lac-Z adenoviral infected cells was determined, there was no specific induction of apoptosis by the bcl-x_S vector in the non-transformed Comma-1D cells, compared to almost a 10 fold specific induction of apoptosis in bcl-x_S adenovirus infected Myc-83 cells (**Table 1**). Thus, the ELISA data suggest that low levels of viability of bcl-x_S infected c-myc transformed mammary cells observed in Figures 3 & 4 is due to induction of apoptosis in these cells by the bcl-x_S vector. In contrast, the Comma-1D cells do not undergo apoptosis or loss of viability in response to bcl-x_S adenoviral infection.

Development of an ascites model for testing therapeutic efficacy of bcl-x_S gene therapy

We previously demonstrated that intralesional injections of the bcl-x_S adenoviral vector into MCF-7 solid tumors in nude mice caused local tumor regression (Ealovega et al., 1996;). This model, however, did not allow for the assessment of therapeutic index or efficacy. Furthermore, gene therapy in solid tumors is limited by problems of viral delivery. In order to more accurately assess the therapeutic index and efficacy of bcl-x_S adenoviral gene therapy we developed a breast carcinoma ascites model. To accomplish this we utilized the MDA-MB-231 human breast carcinoma cell line which grows efficiently as ascites in nude mice.

In vitro experiments demonstrated that the MDA-MB-231 cells were sensitive to bcl-x_S adenoviral induced apoptosis. At an MOI of 2000 on day 6 after infection, the bcl-x_S infected cells had a viability of $32 \pm 6.79\%$ compared to the lac-Z infected cells' viability of $92 \pm 6.36\%$ (n=5). The Cell Death ELISA confirmed that the bcl-x_S vector induced apoptosis under these conditions (data not shown). The delivery of the bcl-x_S adenovirus into the peritoneal cavity of nude mice with ascites derived from MDA-MB-231 cells, permits comparison of the

effects of Bcl-x_S expression in ascites tumor cells compared to normal mouse cells, thus allowing evaluation of the therapeutic index of this agent in vivo.

Intraperitoneal injections of bcl-x_S adenovirus in nude mice produce no detectable toxicity

In order to determine the ability of adenoviral vectors to transduce normal mouse peritoneal cells and to assess the effect of Bcl-xS expression on these cells in vivo, we injected non-tumor bearing nude mice with a mixture of bcl-x_S and lac-Z adenoviruses so as to identify adenoviral transduced protein in cells in situ; and to simultaneously assess the effects of Bcl-xS expression on these normal cells. The animals were injected intraperitoneally with a mixture of bcl-x_S and lac-Z adenoviruses (ratio of 9:1: using 6×10^9 pfu of bcl-x_S virus and 0.60×10^9 pfu of the lac-Z virus), and demonstrated no evidence of local or systemic toxicity (n=3). The animals were sacrificed a week after receiving the viral injection; and abdominal organs were sectioned and stained for β-galactosidase activity. We observed significant X-gal staining in the sections of liver, kidney, and spleen as shown in **Figure 5** (Panels **a**, **b**, and **c** respectively).

The organ sections in Figure 5 showed no evidence of cellular toxicity or cell death. This was confirmed by H & E staining of serial sections of the liver, spleen, kidney, and other abdominal organs (data not shown). Immunohistochemical determination of Bcl-xS protein expression within the organ sections could not be done because all Bcl-x antibodies which recognize the Bcl-xS form of the protein, also recognize Bcl-xL, the other form of the Bcl-x protein. Since Bcl-xL is highly expressed in these organs, specific detection of Bcl-xS immunohistologically, cannot reliably be accomplished with existing reageants.

Efficiency of gene transduction by adenoviral vectors in the ascites model

The above experiments demonstrated that the bcl-x_S adenovirus could be injected intraperitoneally with no apparent toxicity to normal cells. In order to demonstrate the therapeutic index of intraperitoneal injections of bcl-x_S adenovirus, we utilized nude mice bearing ascites derived from MDA-MB-231 breast carcinoma cells. We first determined the efficiency of adenoviral gene transduction in this model by measuring the ability of the lac-Z adenovirus to transduce the ascites cells in vivo. Following a single IP injection of 3×10^9 pfu of the lac-Z vector, cells were aspirated on day three and stained for β-galactosidase activity. At this titer, approximately 40% of the viable cells in the aspirate showed X-gal staining (n=3). In order to demonstrate Bcl-xS

protein expression in transduced cells, we performed western blotting on lysates derived from ascites cells withdrawn from the abdominal cavity three days following administration of the bcl-x_S adenovirus in vivo. As shown in **Figure 6,** Bcl-xS protein was detected in aspirates of ascites from mice which received the bcl-x_S vector (lane 2), but not the lac-Z vector (lane 1). The level of expression of Bcl-x_S in these studies may be an underestimate since cells expressing this protein undergo an apoptotic death.

Therapeutic efficacy of intraperitoneal bcl-x_S gene therapy

We next determined the effects of IP administration of the bcl-x_S adenovirus on ascites formation and survival of nude mice bearing breast carcinoma. The viral titer per injection used for these experiments is one that resulted in Bcl-xS expression in the ascites model in vivo without demonstrated toxicity (3×10^9pfu). Since, we observed Bcl-xS protein expression in mice 3 days following administration of the bcl-x_S vector (Figure 6), we chose a regimen where each mouse received three viral injections of either bcl-x_S or the lac-Z vector once every 4 days.

Relative Weight change (RWC) was used as an indirect measure of ascites formation, as described (Hamilton et al., 1984;). RWC data were analyzed by the mixed effects model, which estimates the rates of relative weight chzange for each group of mice (Littel et al., 1996). The estimated rate of RWC for lac-Z treated and uninjected mice were only marginally different (+ 1.53% per day and + 2.20% per day respectively; p=0.0733). In contrast, the estimated rate of RWC for bcl-x_S treated mice was greatly decreased (- 0.06% per day). Comparison of the estimated rates of RWC for bcl-x_S versus lac-Z treated mice showed that they were highly significantly different (p= 0.0001). Thus, mice injected with the bcl-x_S adenovirus showed a very significant decrease in ascites formation compared to the lac-Z injected mice or uninjected controls.

As a more direct test of the therapeutic efficacy of bcl-x_S adenovirus, we assessed the effects of intraperitoneal bcl-x_S gene therapy on survival of ascites bearing mice. **Figure 7** shows that the median survival time for lac-Z treated and uninjected mice after treatment, were 4 and 5 days respectively. In marked contrast, mice treated with the bcl-x_S vector had a median survival time of 53 days after treatment. The difference in survival time between mice treated with the lac-Z versus the bcl-x_S vectors was highly statistically significant (n=7 per treatment group; p=0.0004). Furthermore, there was no apparent systemic toxicity of intraperitoneal bcl-x_S adenoviral gene therapy.

DISCUSSION

In the normal mammary gland apoptosis is triggered by activation of extracellular proteases which degrade the basement membrane, transmitting an apoptotic signal. In addition, the pro-apoptotic genes bcl-x_S and bax are induced (Heermeier et al., 1996;). Recent discoveries elucidating the role of Bcl-xL and Bcl-2 in inhibiting apoptosis, allow a better understanding of the mechanism of Bcl-xS action. Bcl-xS was thought to induce apoptosis by antagonizing the protective function of Bcl-2 and Bcl-xL. This antagonistic action of Bcl-xS was independent of the ability of Bcl-xS to bind Bcl-xL (Minn et al., 1996). It is now clear that apoptosis is initiated when a death signal triggers cytochrome C release from the mitochonria to the cytosol, resulting in activation of a specific protease termed caspase-3 (Li et al., 1997;). Overexpression of Bcl-2/Bcl-xL can block apoptosis by preventing the release of cytochrome C into the cytosol (Kim et al., 1997;). Conversely, overexpression of Bcl-xS, the Bcl-xL antagonist, inhibits the binding of Bcl-xL to cytochrome C, thus permitting release of this redox enzyme into the cytosol (Kharbanda et al., 1997;). The released cytochrome C is part of a protein complex which results in caspase-3 activation. Specifically, a precursor caspase-9 is activated when bound by a complex containing cytosolic cytochrome C and a "docking" protein termed Apaf-1 (apoptosis protease activiating factor), in the presence of dATP/ATP. The activated caspase-9 activates caspase-3, which in turn leads to the breakdown of structural nuclear proteins and genomic DNA (Li et al., 1997;), characteristic of apoptosis.

We hypothesize that during carcinogenesis, genetic events such as overexpression of Bcl-2 or Bcl-x_L, permit a cell to escape induction of apoptosis, thereby conferring a selective advantage to these cells. This hypothesis is consistent with recently described models of carcinogenesis in the mammary gland and pancreas, in which early hyperplastic lesions demonstrate increases in cell proliferation accompanied by increases in apoptosis (Naik et al., 1996;). In contrast, the progression from hyperplasia to carcinoma is accompanied by marked decrease in apoptosis in carcinoma cells. In a transgenic model of pancreatic carcinogenesis, this decrease in apoptosis results from Bcl-x_L overexpression (Naik et al., 1996;). We postulate that carcinoma cells are more sensitive to Bcl-x_S induced apoptosis than normal cells, since overexpression of Bcl-xL (or Bcl-2) in carcinoma cells is required to block apoptosis, which occurs as a consequence of aberrant expression of genes regulating proliferation (Evan et al., 1995;). Antagonizing Bcl-xL or Bcl-2 by expression of Bcl-xS, triggers these transformed cells to undergo spontaneous apoptosis. In contrast to tumor cells, normal cells require additional apoptotic stimuli to trigger this pathway. This hypothesis is further supported by our finding that the bcl-x_S adeno-

virus is lethal to a variety of carcinoma cells (Clarke et al., 1995).

The data presented in this manuscript directly support the above hypothesis, since c-myc transformed murine mammary cells are markedly more sensitive to apoptosis induced by bcl-x_S adenovirus compared to their non-transformed counterpart. Furthermore, this differential sensitivity is not due to differences in adenoviral infectivity or Bcl-xS protein expression. This data is also consistent with our previous observations that the bcl-x_S adenovirus can select-ively induce apoptosis in mammary carcinoma cells compared to bone marrow progenitors, suggesting that this may be a useful agent for bone marrow purging in autologous transplantation (Clarke et al., 1995;). Direct comparison of the effects of Bcl-xS expression in non-transformed versus c-myc transformed mammary cells provides a better test of this hypothesis since differences in species and tissue types in previous studies might account for differential sensitivity to the adenoviral bcl-x_S vector. Furthermore, this model has direct clinical relevance since up to 30% of human breast carcinomas overexpress c-myc (Berns et al., 1996;), and such tumors may be particularly responsive to Bcl-xS induced cell death in vivo. It remains to be determined whether other genes which are overexpressed in human breast carcinomas, such as cyclin-D, sensitize cells to Bcl-xS induced apoptosis.

We extended the in vitro experiments on sensitivity of normal versus tumor mammary cells to the bcl-x_S adenovirus, to a mouse breast cancer ascites model. We demonstrate that intraperitoneal injections of the bcl-x_S adenovirus reduce ascites formation and markedly increase survival in animals bearing MDA-MB-231 ascites. Furthermore, no systemic or local toxicity was observed, despite the demonstrated ability of adenoviral vectors to transduce normal mouse cells in vivo.

The use of adenoviral vectors bearing death promoting genes to treat intraperitoneal carcinomatosis has been previously reported. Yee et al demonstrated that IP administration of an adenovirus vector encoding the herpes virus thymidine kinase gene (HSV-TK), followed by ganciclovir (GCV) treatment, resulted in a modest increase in survival in a breast cancer ascites model. However, they reported significant toxicity with this approach, including marked cytopathic changes in the liver. These results demonstrated that adenoviral vectors can transduce normal cells in vivo. However, the expression of HSVTK followed by GCV treatment resulted in equival-ent toxicity to normal and tumor cells. In contrast, in our studies the bcl-x_S adenovirus produced more pronounced effects on cell survival with less apparent toxicity, consistent with our in vitro results showing selective killing of c-myc transformed mammary cells compared to non-transformed cells (Yee et al., 1996;).

These data in combination with our previous studies showing that low levels of Bcl-x_S expression sensitize breast cancer cells to chemotherapy induced apoptosis (Sumantran et al., 1995;), and that intralesional injections of the bcl-x_S vector caused tumor regression when administered intralesionally in vivo (Ealovega et al., 1996;); strengthen the rationale for developing the bcl-x_S adenovirus as a gene therapy agent for breast cancer. Furthermore, these results have implications for the development of other strategies to antagonize bcl-2 family members for cancer therapy.

ACKNOWLEDGMENTS

We sincerely thank Dr. Robert Dixon (Lombardi Cancer Center, Georgetown University) for providing the Myc-83 and Comma-1D cells mouse mammary cells. We also thank Mark. W. Ealovega for initiating studies on ascites formation in mice, and Kay Cherian for producing adenoviral stocks. We thank Dr. Gabriel Nunez and Dr. Martin Sanda for helpful discussions.

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TABLE 1

Differential Induction of Apoptosis by bcl-xs adenovirus in normal versus tumor mouse mammary cells

Fold Increase in Apoptosis[a]

Mean Units of cytoplasmic DNA-histone (± S.E.M.)[b]

CELL TYPE	control	lac-Z infected	bcl-xs infected	Fold Increase in Apoptosis	
				bcl-xs/control	bcl-xs/lac-Z
COMMA-1D	0.14 (0.005)	0.16 (0.05)	0.25 (0.08)	1.78	1.59
MYC-83	0.08 (0.008)	0.15 (0.03)	1.61 (0.39)	21.47	11.11

Comma-1D normal cells and the Myc-83 cells were infected with the Adv-bcl-xs or Adv-lac-Z vectors (MOI of 1000). Two days post infection cytoplasmic extrtacts were isolated from each sample. The extracts were normalized for total cell number (3×10^4 cells/mL) and quantitated for DNA-histone complexes by the Cell Death ELISA. The data show units of DNA-histone in each sample. Fold increase in apoptosis represents the amount of DNA-histone in the bcl-xs infected cells relative to uninfected cells or lac-Z infected cells (n=3 and S.E.M is shown). The level of DNA-histone in bcl-xs infected Myc-83 cells was significantly greater than that in bcl-xs infected Comma-1D cells (p=0.03).

[a] Fold Increase in apoptosis = $\dfrac{\text{Units of DNA-histone in treated cells}}{\text{Units of DNA-histone in untreated cells}}$

[b] 1.0 $\text{Unit}_{405\ nm}$ = peroxidase activity conjugated to anti-DNA-anti-histone antibodies, specifically bound to a cytoplasmic extract from 2×10^3 HL-60 cells treated with camptothecin (2 µg/mL for 4 hours).

FIGURE LEGENDS

Figure 1: Adenoviral Infectivity of Comma-1D and Myc-83 cells: After 2 days of infection with the lac-Z adenoviral vector (MOI of 1000), the lac-Z infected cells were assayed for β-galactosidase activity. In this experiment approximately 70% of the Comma-1 D cells (Panel *A*) and Myc 83 (Panel *B*) cells infected with the lac-Z vector, expressed β-galactosidase activity. The average percent of Comma-1 D cells cells expressing lac-Z was 76 % ± 4.60 %, whereas the Myc-83 cells showed an average lac-Z expression of 81% ± 3.20 % under these infection conditions (n=3, ± S.E.M.).

Figure 2: Western blot demonstrating Bcl-xS protein expression: Comma-1D and Myc 83 cells were infected with either the bcl-x_S or lac-Z adenoviral vectors (MOI of 1000). Two days after infection, lysates were prepared and electrophoresed on 12% SDS-PAGE gels as described. The blot was probed with antibody to human Bcl-x. The Bcl-xS protein is indicated by the arrow. Lanes 1,2, and 3 represent lysates of uninfected Comma-1D cells, Comma-1D cells infected with bcl-x_S virus, and lac-Z virus respectively. Lanes 4,5, and 6 represent lysates of uninfected Myc-83 cells, Myc-83 cells infected with the bcl-x_S, and lac-Z adenoviruses respectively.

Figure 3: Effect of bcl-x_S adenovirus on cell morphology: Comma-1D normal cells and the Myc 83 mouse tumor cell types were infected with the bcl-x_S or lac Z vectors (MOI of 250) and photograhed 4 days later. Panel*a* : uninfected Comma-1D cells. Panels *b* and *c* : Comma-1D cells infected with the lac-Z and bcl-x_S viral vectors respectively. Panel*d*. : Uninfected Myc-83 cells. Panels*e* and*f* : Myc-83 cells infected with the lac-Z and bcl-x_S viral vectors respectively.

Figure 4: Effect of bcl-x_S adenovirus on cell viability: Comma-1D mouse cells and the c-myc overexpressing mouse mammary tumor cell line (Myc 83), were infected with the bcl-x_S or lac-Z adenoviral vectors (MOI of 1000). Two days after infection cells from duplicate samples were counted. The graph shows dead cells as a percentage of total cell number in each sample (n=3, S.E.M. is shown). The difference in viability of the bcl-x_S infected Myc-83 cells versus bcl-x_S infected Comma-1D cells was highly significant (p=0.003).

FIGURE LEGENDS (CONTINUED)

Figure 5: Effect of intraperitoneal administration of bcl-x_S and lac-Z adenoviruses on normal histology of nude mice: Three normal nude mice were injected with a mixture of bcl-x_S and lac-Z adenoviruses (ratio of 9:1, total viral titer = 6.60×10^9) to identify adenoviral infected cells in situ. The mice were sacrificed after 7 days, and abdominal organs were cryopreserved, sectioned, and stained for β-galactosidase activity. Data from all 3 mice were consistent. Significant levels of X-gal staining were observed in the liver, kidney, and spleen, with no apparent cytopathic effect (Panels ***a***, ***b***, and***c*** respectively).

Figure 6: Bcl-xS protein expression in vivo: Nude mice bearing MDA-MB-231 ascites were injected once intraperitoneally with either the bcl-x_S or lac-Z viral vector (total viral titer = 3×10^9). After 3 days, ascites cells from each mouse were aspirated, plated, and harvested for SDS-PAGE analysis as described. Western blotting showed that the Bcl-xS protein (see arrow) was detected in the aspirate from the mouse injected with the bcl-x_S adenoviral vector (Lane 2), but not in the aspirate from the mouse injected with the lac-Z vector (Lane 1).

Figure 7: Effect of bcl-xs gene therapy on survival of ascites bearing mice: Nude Mice with MDA-MB-231 carcinoma ascites were divided into three groups (Bcl-xS treated, Lac-Z treated, or untreated controls). Three viral injections of either bcl-x_S or the lac-Z adenovirus vector were given once every 4 days, and occurred on days 0, 4, and 8 respectively. Survival distributions are displayed using the Kaplan-Meir method (n=7 per group).

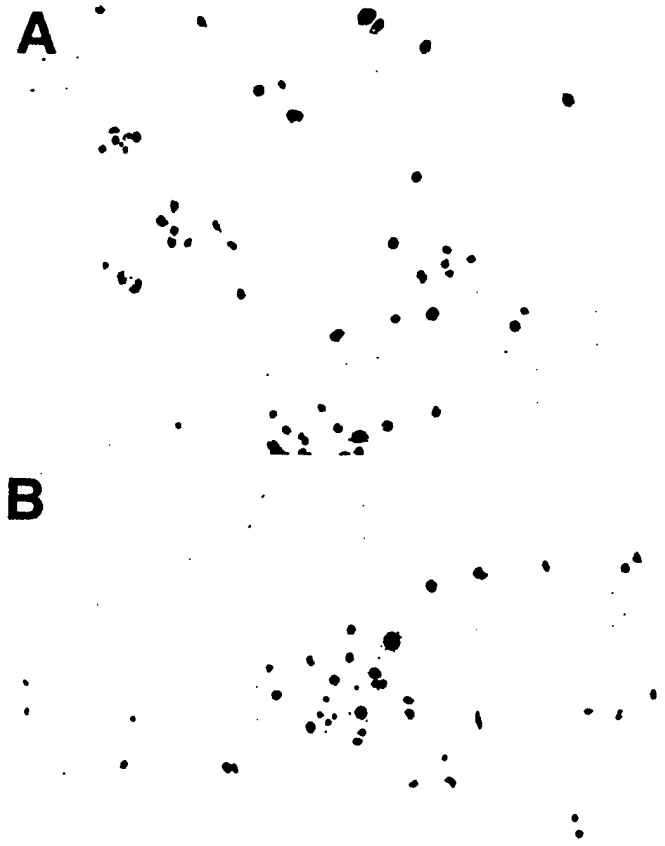

Figure 1

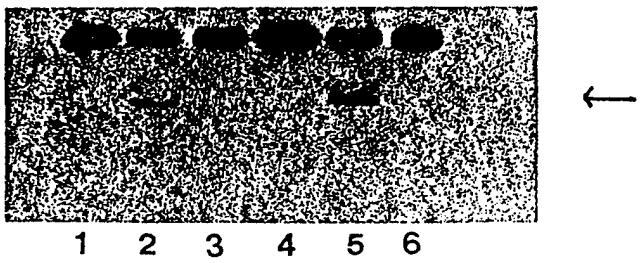

Figure 2

Figure 3

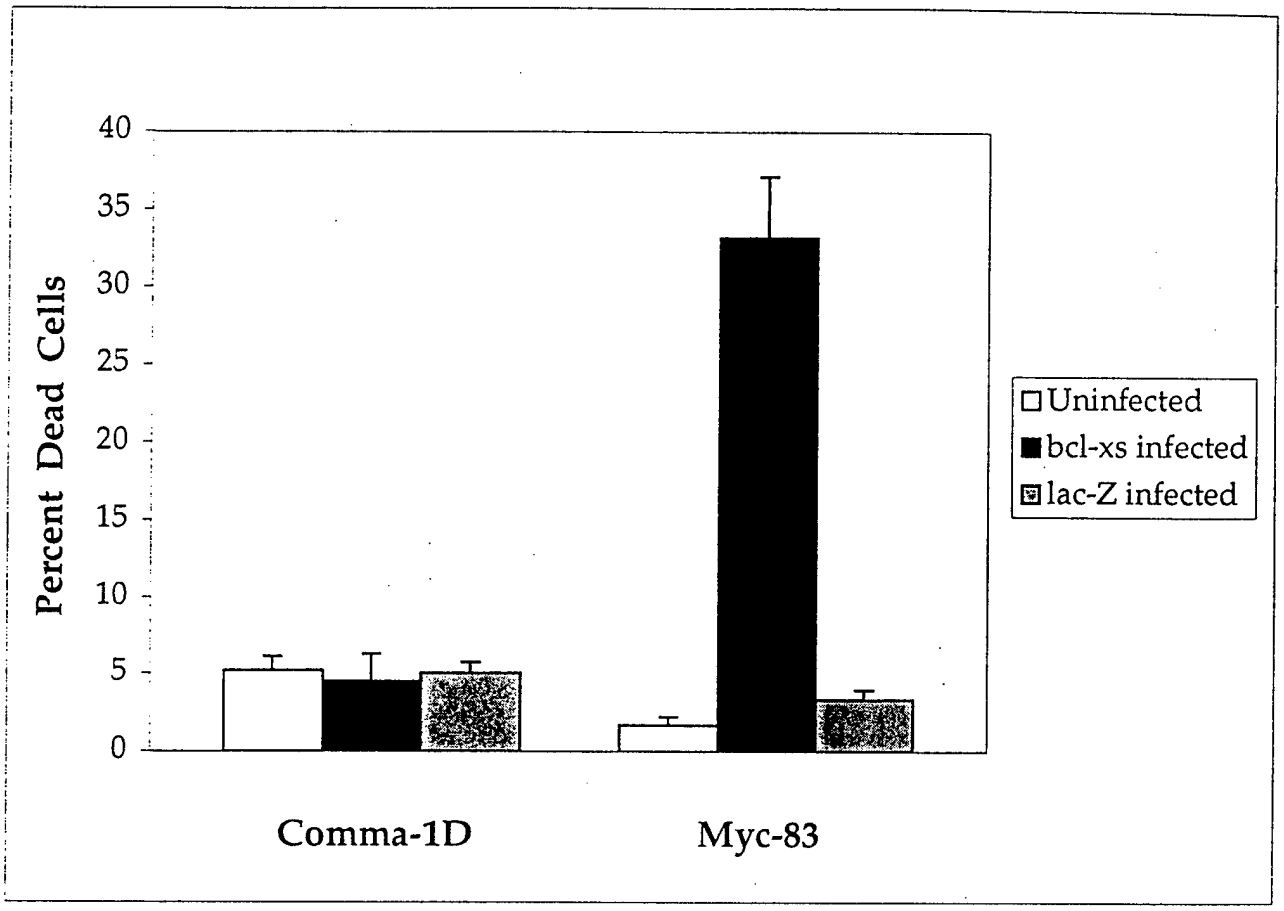
Percent Dead Cells
40
35
30
25
20
15
10
5
0
Comma-1D
Myc-83
Uninfected
bcl-xs infected
lac-Z infected

Figure 4

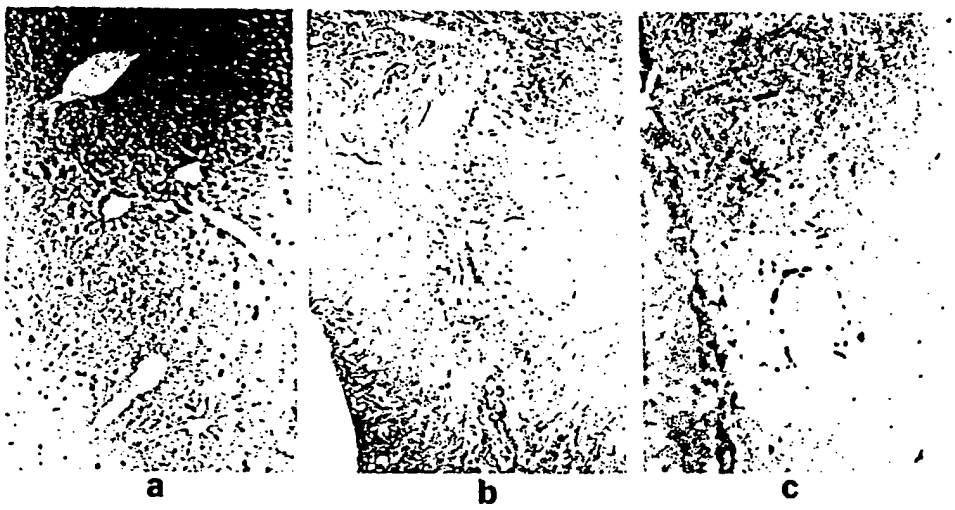

Figure 5

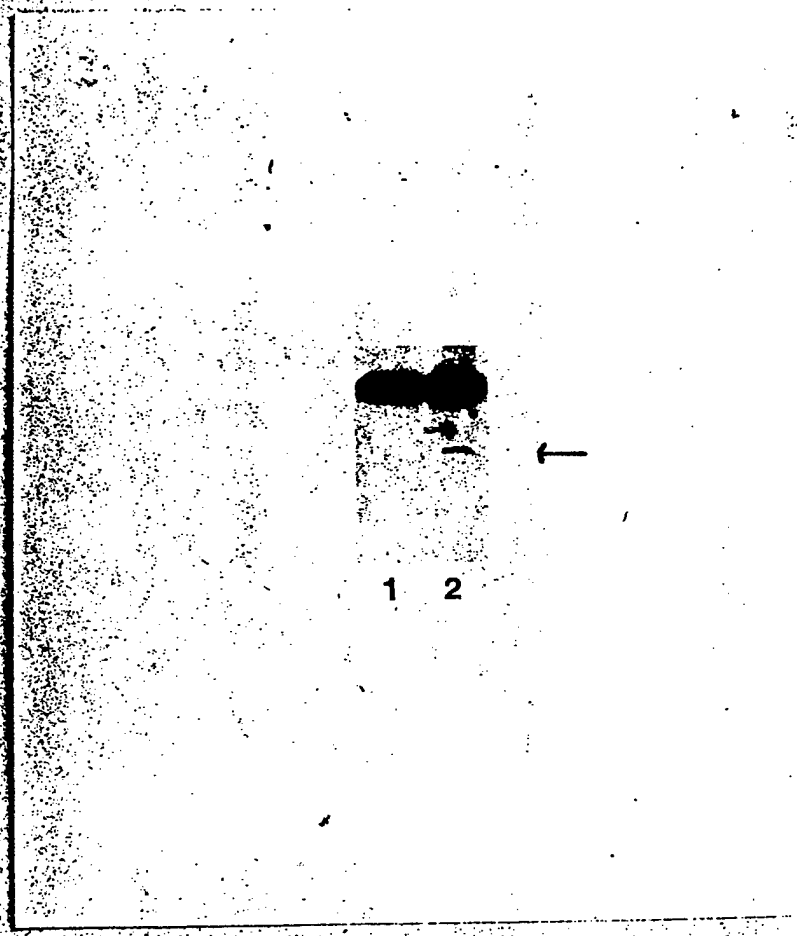

Figure 6

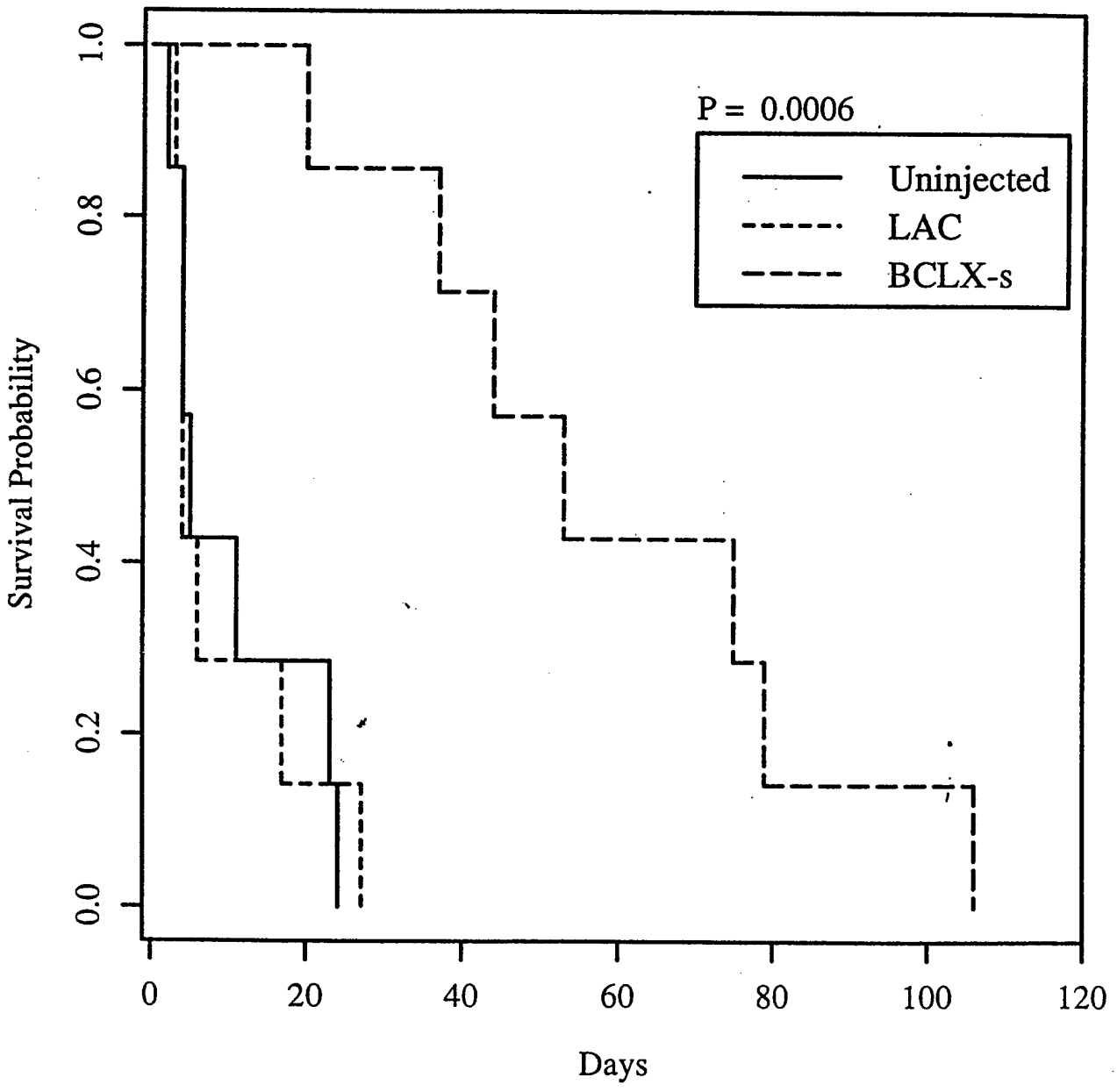
Survival by Treatment
P = 0.0006
Uninjected
LAC
BCLX-s
Survival Probability
0.0
0.2
0.4
0.6
0.8
1.0
0
20
40
60
80
100
120
Days

Figure 7

Springer Semin Immunopathol (1997) ♥♥ : ♥♥♥–♥♥♥

Springer Seminars
in Immunopathology
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Targeting cancer cell death with a bcl-x_S adenovirus

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Introduction

Recent advances on the molecular basis of cancer have indicated that transformation is not solely a matter of increased cellular proliferation [60]. It has become widely accepted that more than one genetic alteration is necessary for a cell to become cancerous [80]. This is due to built-in cellular mechanisms that can detect abnormalities and activate genetic programs to remedy them, making single adverse events less likely to cause significant problems. For example, tumor suppressor genes are best known for their ability to halt cell cycle progression and allow time for repair and maintenance of genomic integrity [83]. Malfunction of this system constitutes a second defect that can lead to malignant transformation [80].

More recently, a second protective mechanism known as apoptosis has been identified as important in deleting potentially dangerous cells from an organism [28]. Programmed cell death (PCD) is a genetic program that initiates a series of events resulting in cell suicide. This process leads to apoptosis, a morphologically distinct form of death with physical characteristics such as nuclear fragmentation, membrane blebbing, and DNA degradation [86]. Apoptosis plays a vital role in the normal development of an organism. Its many functions include regression of the tadpole tail in frogs [44] as well as the removal of self reactive lymphocytes in the formation of mammalian immunity [72].

DNA damage and the improper expression of oncogenes have been shown to induce programmed cell death [28]. This is presumably a safeguard against cancer. Genetic defects that permit a cell to constitutively block apoptosis confer a selective growth advantage to that cell. Thus, it makes sense that this is an essential step in the progression to cancer. The discovery and characterization of several apoptosis modulators have verified that this is indeed often the case. Improper regulation of these genes can increase cell survival and provide tumor resistance to traditional forms of cancer treatment (radiation and chemotherapy) that function by activating PCD [32]. This generally leads to poor clinical prognosis.

Fortunately, these same pathways that block cell death represent a potential target for the rational design of new therapies. In this review, we will introduce the factors

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Springer Semin Immunopathol
Artikel: **539**
Schneider Druck GmbH, Rothenburg

Springer, Heidelberg
Seite(n): **1–10**
1. Korrektur: 23.10.97

Bearbeitung:
Springer, EB

regulating apoptosis in cancer and discuss possible areas for intervention. We will also provide an example of such intervention, in which an adenovirus is used to deliver a pro-apoptotic gene, *bcl-x*$_S$.

Programmed cell death

PCD is best understood in the nematode *Caenorhabditis elegans*, where the fate of all 1090 cells have been tracked. Of these cells 131 undergo cell death during development, and genetic studies revealed the master controls behind this phenomenon. A gain of a function mutant of the *ced*-9 (*ced* for cell death abnormal) gene caused all 131 cells that are normally eliminated to survive, while loss of function *ced*-9 mutations caused more than the usual 131 cells to die [38]. In contrast to *ced*-9, the *ced*-3 and *ced*-4 genes cause cell death [27]. In *ced*-4;*ced*-9 and *ced*-3;*ced*-9 double mutants all cells live, which suggests that Ced-3 and Ced-4 are cell death effectors, and Ced-9, which functions upstream, is an antagonist of these proteins. It is known that Ced-4 binds to Ced-3 resulting in activation of the caspase [42, 85]. Ced-9 binds to Ced-4, blocking its ability to activate Ced-3 [73, 85]. This apoptosis machinery has been well conserved throughout evolution. We now know that Bcl-2 family members are mammalian homologues of Ced-9 [39] and the caspases (or ICE proteases) are homologues of Ced-3 [90]. No homologue of Ced-4 has yet been identified, but the apparent interchangability of these proteins between nematode and mammalian systems has facilitated the ordering of these genes [14, 15, 84].

Improper growth stimulation leads to apoptosis

The expression of key genes is involved in the signal to proliferate. Due to their mitogenic properties many of these are proto-oncogenes and have been implicated in human carcinogenesis, since uncontrolled growth is one of several steps along the path towards cancer. Recent evidence suggests that inappropriate expression of these genes leads not only to proliferation but to activation of the PCD pathway.

Deregulated expression of the c-*myc* gene occurs in up to 30% of human cancers [61]. In normal cells, expression of the c-*myc* proto-oncogene is rapidly up-regulated when a cell initiates proliferation [2]. Although all of the functions of c-Myc are not completely understood, it is a transcription factor that, when highly expressed, is able to overcome growth arrest and to block differentiation [5, 6, 25, 62]. Interestingly, c-Myc, whose expression is so tightly linked with cell growth, has also been shown to induce cell death. When c-Myc is highly expressed in cells that are deprived of growth factor, they undergo apoptosis [29]. Therefore, the consequences of c-Myc expression depends on the context of other proliferative signals.

A similar story holds true for the c-*fos*/*jun* proto-oncogenes. Their protein products associate to form a transcription factor called activator protein-1 (AP-1) [17]. Like c-Myc, AP-1 is induced upon mitogenic stimulation and appears to be involved in mediating cell cycle progression [1]. c-Fos and Jun expression have also been correlated with PCD in response to unfavorable growth conditions or cell injury [10, 23]. Further implicating c-Fos in apoptosis were experiments demonstrating that ectopic expression of c-Fos led to cell death under conditions in which cells were normally quiescent [63]. The converse experiment (inhibition of c-Fos expression) increased cell survival under

conditions which normally led to mass apoptosis [23]. This supports the hypothesis that c-Fos somehow regulates apoptosis.

At first these two opposing functions of both c-Myc and AP-1 may appear to be contradictory. However, closer scrutiny reveals that induction of apoptosis by growth effectors may be an important safety means by which proliferation can be halted if such factors are expressed at inappropriate times. This can selectively eliminate cells with potentially carcinogenic alterations, which often result in proliferation regardless of the external signals provided by their environment. The mechanism by which this takes place is as yet unknown. It is possible that the proto-oncogenes that induce proliferation also inherently produce a continuous death signal that can only be stopped under favorable growth conditions. For example, certain growth factors apparently function to inhibit the cell death pathway [12, 21, 34]. On the other hand, apoptosis may be the result of conflicting growth and quiescence signals within the same cell.

In addition, some tumor suppressor genes also function by regulation of PCD. One of the most common abnormalities in human malignancy is a mutation of the *p53* tumor suppressor gene [79]. Up to 50% of human cancers harbor such mutations. p53 exerts its tumor-suppressing effects in two ways: cell cycle regulation and apoptosis. p53 controls the cell cycle through transcriptional activity. Putative p53 DNA binding sites have been identified and can direct mRNA synthesis upon activation of p53 [43, 64]. Several genes have thus far been shown to be under the direct transcriptional control of p53. One of these, *p21* (also called *waf1* or *cip*-1) is activated by p53 in response to cellular damage [26]. Elevated levels of p21 can cause growth arrest by binding to cyclin-Cdk complexes and inhibiting kinase activity [51]. In addition, p53 has been implicated in the repair of radiation-induced DNA damage and transcriptional repression [49, 70].

Perhaps more importantly, p53 has been shown to induce apoptosis under several conditions. Sometimes the simple restoration of p53 in a transformed cell is enough to cause cell death [89]. In another example, mouse thymocytes lacking p53 are resistant to apoptosis caused by radiation and various forms of chemotherapy, while p53-positive cells die when treated in the same way [18, 52]. This suggests that p53 is a major downstream effector of current methods of cancer treatment.

The ability of p53 to induce apoptosis usually does not require transcription, and some p53 mutants that cannot bind DNA and stimulate RNA synthesis are still able to activate the cell death pathway [36, 82]. Therefore, in some systems the apoptotic function of p53 can be separated from the cell cycle regulation, which does require transcription. However, it should be noted that p53-mediated apoptosis is not always transcription independent. Some studies indicate that p53 promotion of cell death can be transcription dependent [67]. This is further supported by the finding that the Bcl-2 family member (see below) Bax, which is pro-apoptotic, has a promoter containing p53 binding sites and is transcriptionally activated by p53 [55].

Members of the bcl-2 family regulate apoptosis

The *bcl*-2 gene was discovered at the breakpoint of a t(14;18) translocation that commonly occurs in B cell lymphomas [20, 76]. Bcl-2 was shown in culture to suppress apoptosis normally induced by a variety of factors including growth factor withdrawal, γ-irradiation, and chemotherapeutic drugs [58, 69]. The protective effects of Bcl-2 are not universal, as T cell deletion still occurs in the presence of Bcl-2 overexpression

[69]. When constitutively expressed in transgenic mice, Bcl-2 led to an accumulation of B cells [53]. The expansion of the B cell population was not due to enhanced cell proliferation but to decreased cell death. The enhanced B cell life presumably allowed secondary genetic abnormalities to accumulate, and eventually ended in lymphoma. Further investigation demonstrated that Bcl-2 knockout mice were subject to increased apoptosis and loss of mature lymphocytes [78]. Nevertheless, these mice were able to survive through development, implying a functional redundancy with respect to Bcl-2.

During the past few years several homologues of Bcl-2 have been identified. Members of this family of proteins contain one or more of four Bcl-2 homology (BH) regions termed BH1, BH2, BH3, and BH4. Bax, Bad, Bak and Bik negatively regulate apoptosis, apparently by antagonizing Bcl-2 [8, 16, 31, 46, 59, 87]. Another Bcl-2 family member, Bcl-x, can be present in one of two forms depending on how the primary RNA transcript is spliced [7]. The larger of the two Bcl-x proteins, Bcl-x_L, contains all four BH regions and exhibits the highest homology to Bcl-2. In culture Bcl-x_L displays remarkable similarity to Bcl-2 in ability to block apoptotic response to a range of external signals. The smaller protein, Bcl-x_S, contains BH3 and BH4 regions and can actually accelerate apoptosis in certain situations, such as cytokine withdrawal from interleukin-3-dependent cell lines. In addition, Bcl-x_S abrogates the protective functions of both Bcl-2 and Bcl-x_L. The relative levels of Bcl-x_L and Bcl-x_S appears to be an important factor in cell survival.

The actual mechanism by which Bcl-2 family members carry out their actions is an area of intense investigation. The carboxy terminus of Bcl-2 contains a hydrophobic transmembrane domain that localizes Bcl-2 primarily to the outer mitochondrial membrane [48]. Removal of this targeting domain from Bcl-2 and related family members either abolishes or diminishes protective activity, which implies that membrane localization is important for Bcl-2 function [57, 75]. It is likely that Bcl-2 acts by inactivating cell death effectors such as a mammalian version of Ced-4 [15, 84]. In other theories Bcl-2 has been postulated to act by controlling the cytoplasmic level of intercellular species such as p53, Cdks, cytochrome *c*, Ca^{2+}, or reactive oxygen species [3, 41, 47, 50, 54, 66, 88]. How all of these processes relate is still poorly understood.

Apoptosis regulators – who is really in control?

So far we have discussed the c-Myc, AP-1, p53, and Bcl-2 proteins in separate contexts. However, it is obvious that proteins playing such critical roles in the cell must have some degree of interdependence. It is unlikely that there would be so many autonomous apoptosis pathways. A more likely scenario is the existence of multiple ways in which apoptosis can be triggered, all of which converge upon a group of central regulators.

p53 has been shown to activate an apoptosis program not only in response to damage caused by external agents, but also in response to internal cellular dysfunction. This raises the question of whether improper expression of genes such as c-*myc* and *fos*/*jun* induce apoptosis in a p53-dependent fashion. Evidence exists that this is the case. c-Myc-induced apoptosis is not apparent in several cell lines devoid of functional p53, but is restored upon the introduction of wild-type p53 [40, 82]. Similar experiments have shown that this p53 dependence also holds true with c-Fos [63]. Therefore, it appears that p53 acts downstream of Myc and Fos and at least in some cases is an intermediate through which the Myc and Fos cell death signals act.

Bcl-2 and Bcl-x_L can inhibit both c-Myc- and p53-induced apoptosis [4, 30, 33, 68, 77, 81]. This, in addition to the multitude of other death signals that can be antagonized by Bcl-2, suggests that the Bcl-2 family acts downstream of most apoptosis effectors and is one of the final resorts in stopping PCD. Because alteration of Bcl-2 family regulation can block most forms of apoptosis, this represents an efficient manner in which cells could become transformed. Thus, it is logical that Bcl-2 family members might play a role in many cancers. It is currently thought that in up to 60% of all cancers, apoptosis is inhibited through overexpression of a Bcl-2 family member [11, 13, 22, 24, 71].

Therapeutic targeting of apoptosis pathways in transformed cells

Our increased understanding of the hierarchical ordering of apoptosis regulators may be useful in targeting treatment of transformed cells. Since it appears that the majority of cell death pathways converge and are under the control of Bcl-2/Bcl-x_L, negative regulators of Bcl-2 and Bcl-x_L would probably allow a cell to act upon apoptotic signals. The delivery of such a gene to cancerous cells would relieve the protection provided by elevated expression of an apoptosis inhibitor. High expression alone might kill the cells, and lower expression levels could increase cellular sensitivity to radiation or chemotherapy.

To test this hypothesis, our laboratory constructed a recombinant, replication-incompetent adenovirus vector expressing the *bcl-x_S* gene, a functional inhibitor of Bcl-2 and Bcl-x_L [19]. This vector was able to efficiently introduce the gene into a wide variety of cell lines and deliver high levels of expression. As expected, virtually all epithelial-derived transformed cell lines that we have tested to date are killed by the *bcl-x_S* virus via apoptosis. Cancer cells derived from patients with neuroblastoma, kaposi's sarcoma, and breast, colon, ovarian, and head and neck cancers all undergo apoptosis when cells express high levels of Bcl-x_S protein [19]. This is true both for primary cancer cells and established, transformed cell lines. Low level expression of Bcl-x_S sensitizes cells to both chemotherapy [74] and radiation therapy. On the other hand, normal fibroblasts and hematopoietic stem cells are relatively resistant to *bcl-x_S* adenovirus-induced apoptosis.

The use of an adenovirus to deliver *bcl-x_S* has potential clinical utility. Results from our laboratories indicate that resistance of hematopoietic stem cells to the virus is at least in part due to the inability to infect such cells. High-dose chemotherapy and autologous hematopoietic stem cell transplantation is increasingly being used to treat both breast cancer and childhood neuroblastoma [35, 45]. Unfortunately, the autologous stem cells used to rescue the patient from lethal doses of chemotherapy are frequently contaminated with cancer cells [9, 37, 65]. The selective killing of the cancer cells by cytotoxic adenovirus vectors makes such agents ideal for eliminating cancer cells contaminating the stem cells collected for re-infusion. Studies from our laboratories have demonstrated the feasibility of this approach. The *bcl-x_S* adenovirus was able to eliminate 1.5×10^4 cancer cells contaminating 10^6 normal bone marrow cells, whereas the normal human hematopoietic stem cells exposed to the virus were still capable of engrafting the bone marrow of SCID mice [19].

This virus may also be useful for the treatment of cancers in other settings. Because non-replicating viruses will only diffuse for limited distances in solid tissues, they can best be delivered to cells in a cavity. Two diseases in which this virus may be useful

are bladder and ovarian cancer. Early bladder cancer arises in the bladder and ovarian cancer initially spreads in the peritoneal cavity. Furthermore, early bladder cancers are superficial tumors which arise focally or diffusely initially penetrating only a few cell layers of the bladder luminal epithelium. Similarly, early in the course of ovarian cancer, or after initial chemotherapy, there are often microscopic foci of tumor cells remaining. In both of these cases, it is quite possible that all of the cells can be infected and killed by an adenovirus vector.

One area of concern is whether normal, non-transformed cells will be adversely affected by the introduction of Bcl-x_S overexpression. Circumstantial evidence from our laboratory indicates that the virus will preferentially kill transformed cells. This suggests that Bcl-x_S itself does not cause apoptosis. Rather, Bcl-x_S may function by allowing other stimuli to initiate an apoptotic pathway without the interference of Bcl-2 family members. Since transformed cells, by virtue of their genetic lesions, are more likely to deliver these signals than their normal counterparts this may explain the selectively of Bcl-x_S-mediated killing. This mechanism will only become clearer when we better understand the mode of Bcl-x_S action and the role of the Bcl-2 family in survival of normal and transformed cells.

Summary

Transformation is a complex cellular process that requires several genetic abnormalities. In many cases, one of these abnormalities is an inhibition of PCD, which provides a selective advantage for tumor cells. This has been recently shown in an in vivo model, where overexpression of Bcl-x_L is a crucial step in the progression from hyperplasia to neoplasia and is accompanied by a significant decrease in tumor apoptosis [56].

Frequently, overexpression of a member of the Bcl-2 family results in a block in cell death and appears to nullify many built-in cellular defense mechanisms against cancer. Such a block presents a problem because radiation and chemotherapy, standard cancer treatments, ultimately exert their effect by induction of apoptosis and would also be made less effective. Therefore, to better treat cancer it may be necessary to develop novel methods to overcome the effects of the Bcl-2 family. One way to approach this problem is to target the cause – the molecular machinery that allows a cancer cell to survive. Advances in our understanding of apoptosis has identified the Bcl-2 family as a mediator of most apoptosis pathways, including those initiated by oncogenes, tumor suppressor genes, growth factor withdrawal, and external damaging signals. Therefore, functional inhibition of Bcl-2 family members is lethal to many cancer cells. Using gene transfer technology, we can now deliver genes that accomplish this goal. Further investigation will reveal whether this translates to improved therapy in the future.

Acknowledgements. This work was supported by grants 5 R55 CA67140-02, CA70057, and 5R01 CA61777-04 from the National Institutes of Health; by Department of Defense grant 941407, and by a grant from the U.S. Army Medical Research Command DAMD17-96–6019.

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